BABOON VAGINAL MICROBIOTA: AN OVERLOOKED ASPECT OF PRIMATE PHYSIOLOGY

BY

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DISSERTATION

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ABSTRACT

The bacterial population of the vaginal canal is a primate infant's first exposure to the microbial community inhabiting the outside world. Yet, little is known about this community and the effect it might have on the development and survival of the infant. Humans and *Papio* baboons share considerable anatomical and physiological similarities in their reproductive tracts. Accordingly, we might expect that the vaginal microbiota of baboons would be similar to that of humans. To characterize the vaginal microbiota of a nonhuman primate, we used denaturing gradient gel electrophoresis (DGGE) to evaluate variations in the vaginal microbiota of a group of 35 baboons housed in a facility where they shared the same diet and the same environmental conditions. We also used a 16S rRNA phylogenetic approach to assess the composition of the baboon vaginal microbiota in a subset of animals from this facility and from the wild. We found that despite the uniform environment, there were appreciable differences in the composition of the microbiota from one individual to another in the captive subjects. Our results also indicate that a simple swab test is sufficient for sampling of the vaginal microbiota in the field, a finding that should help make future, more detailed characterization of the microbiota of wild primates feasible. Previous human vaginal microbiota studies have shown that Firmicutes (mostly *Lactobacillus spp*.) predominate in the human vagina, with Actinobacteria (*Gardnerella vaginalis*) and Proteobacteria present in lower numbers. By contrast, Papio baboons harbored species not only of Firmicutes but also of

Fusobacteria, Bacteroidetes and Spirochaetes phyla that are not normally abundant in humans. Moreover, the Firmicutes found in baboons were different from those found in humans, consisting mainly of clostridia rather than lactobacilli. A further level of difference was also seen within the same phylogenetic groups where baboon bacterial species clustered separately from those reported in humans. Results of our analyses imply that co-evolution of microbes and hosts cannot account for the major differences between the microbiota of baboons and that of humans, because divergences between the major genera were too ancient to have occurred since primates appeared. Instead, the primate vaginal tracts appear to have acquired discrete subsets of bacteria from the vast diversity of bacteria available in the environment and established a community responsive to and compatible with host species physiology.

To my family,

Their superlative love and support

is the lighthouse that guides me to shore.

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As I became a member of the microbiology department in that, proverbial state of professional infancy many new graduate students possess, I came to realize the many challenges lying ahead of me. Idealistic, determined and focus, like any new comer, I began to walk the path with haste on a mission to impress everyone. Soon I was humbled by the attitude and simplicity that the staff and faculty of the department of microbiology displayed. The extraordinary wealth of knowledge and creativity this department possess quickly delivered my first lesson; slowdown, breathe and absorb what they have to teach. And so my graduate adventure began.

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CHAPTER 1

INTRODUCTION

THE MICROBIOTA CONCEPT

Animals live in association with complex and dynamic microbial communities. These microbes colonize numerous host sites, including the skin, mouth, gastrointestinal tract, and vaginal tract. Interactions between the host and these microbes probably exert a major influence on host physiology. The best-understood microbiota effects on the human host occur in the mouth and intestinal tract. The bacterial constituents of the mouth are involved in the formation of dental caries and can be a factor in periodontal disease, depending on the community structure and host health state (6, 20). In the case of the microbiota of the human colon, dietary polysaccharides that are not digestible by the human body are converted into short chain fatty acids by fermentation. These products serve as carbon and energy sources for host cells (34, 74, 96). This microbiota is also thought to protect against pathogens. In contrast to these niches, and perhaps other anatomical sites, the effects of the vaginal microbiota are less well understood. Clearly this bacterial population protects against pathogens that could cause vaginitis and vaginosis. In the case of nonhuman primate females, the vaginal microbiota is thought to influence general health, fecundity and pregnancy outcomes (48, 49, 56, 59, 65, 98, 99, 114, 121). The vaginal microbiota of nonhuman primates is the focus of this thesis.

Recent studies of the human vaginal microbiota, using cultivation-independent methods, found not only tremendous diversity within an individual's vaginal microbiota but also considerable person-to-person variation (3, 58), implying that women tolerate a wide range of microbial co-inhabitants. Such diversity suggests that hosts realize fitness benefits from these relationships.

Host-microbe relationships have been appreciated since the beginning of modern medicine and it is not until recently that these interactions are beginning to be elucidated with culture and culture independent molecular techniques. Prior to the work described in this thesis, nothing had been done using culture-independent methods to assess the microbial composition of the nonhuman primate vaginal microbiota.

In humans, both culture-dependent and culture-independent methods have shown that the microbiota of the human body consists of a complex array of bacterial species. To date, results suggest that the densest population resides in the human intestinal tract, reaching densities of 10^{14} cells/ml in the lumen of the colon (66, 67, 117). Microorganisms, particularly bacteria, were identified in the vagina as early as 1928, but they were not recognized as part of the normal vaginal environment until 1955 (41).

Today we understand that an immense range of different microorganisms coexist in the healthy vaginal canal. The vaginal environment varies by reproductive status, sexual activity, birth control methods, and interventions such as douching and antibiotic use, can disturb this healthy ecology (52). It has been clearly established that disruptions in the delicate balance of the microbial

ecosystem in the vagina result in significant health consequences, including enhanced acquisition of sexually transmitted pathogens, such as HIV, perinatal complications and pelvic inflammatory disease (112). In addition, there are major reproductive implications from disturbing the healthy vaginal environment, including an increase risk for reproductive and in-vitro fertilization failures, and preterm birth.

NONHUMAN PRIMATE MODELS OF WOMEN'S HEALTH

Animal models are a fundamental component of biomedical research. Over the past few decades, an overwhelming majority of critically important research in the field of medicine and related sciences has involved considerable work utilizing animal models. It is clear that information provided through the use of these models, and in particular nonhuman primates, will be essential for continued development of effective solutions to issues affecting the health and well being of both human and animal populations.

Although nonhuman primates represent less than 0.3% of the animals used in biomedical research they have served as important models for the study of human health (40, 101). A large number of fields benefit from the used of nonhuman primates, especially the areas of functional genomics, infectious disease, reproductive biology (including menopause and ovarian function), endocrinology, skeletal health, coronary heart disease, even the effect of stress and diet on obesity (1, 61). The criterion for these models to be considered appropriate is that there are close phenotypic and physiological similarities to

humans, but more importantly on the high percentage genetic coding sequence identity between humans and nonhuman primates. For example, chimpanzees are ~98.5% (great apes) and macaques ~95% (old world monkeys) similar in overall genetic sequence identity to humans (16, 70, 102, 103). The baboon genome remains in the sequencing stages and an overall genetic picture is yet to be determined. However, independent genetic studies reveal high percent identity, ranging from 86% - 98% identity to humans in particular segments of the baboon genome (19, 100, 120).

Reproductive system

Macaques and apes share a similar menstrual cycle with the human female. In the cycle the endometrium is shed periodically, rather than absorbed as in the estrous cycle of rats and mice. The menstrual cycle of macaques, chimpanzees and baboons are similar to that of women in length, variability and cyclic patterns of sex hormones and gonadotropins (71, 82, 107, 108, 122). Therefore, is not surprising that some disorders of the reproductive system are common to these primate species. The similarities of the reproductive system between human and nonhuman primates may contribute to similarities in reproductive tissue responses to environmental factors, namely, bacterial colonization.

The use of nonhuman primate reproductive tract models requires a thorough understanding of the resident microbiota in these animals. The potential for microbial influences over several aspects of nonhuman and human primate

biomedical research outcomes and health is a reasonable postulation. Yet many of these studies lack the evidence to demonstrate the phenomena or have completely ignored the possibility. There have been a number of reports regarding vaginal microbial community structure but the great majority focuses on human vaginal tract. Previous attempts at defining the nonhuman primate microbial content of the vaginal tract have been preformed on baboons, chimpanzees and other primates but these studies are all culture dependent and their results carry the significant bias that is intrinsic in these methods.

Health and disease

Since their inception, microbiology and immunology have provided important information that has had direct application to women's health. There is a critical void however in the area of vaginal ecology. Although the connection between abnormal vaginal ecology and adverse health outcomes has been established, sophisticated tools to assess the vaginal microbial makeup have been grossly lacking until recently. The vaginal microbiota of the human female is dominated mainly by *Lactobacillus* species (22, 38, 54, 75, 91, 97). This association possesses a symbiotic nature since these species have been suspected of preventing disease and maintaining the stability of that environment. Human pathology is often manifested as a shift in their vaginal microbiota from lactobacilli to a polymicrobial environment (28). Such disruptions can impose a high cost to the host as *Lactobacillus spp.* prevalence is inversely related to adverse pregnancy outcomes and ultimately reproductive failure. At

present, the best recognized shift in vaginal microbial composition, from normal to abnormal flora, is reported as bacterial vaginosis (BV). BV during pregnancy is associated with preterm labor and early pregnancy loss. While the association of preterm birth with infection and inflammation has been compelling, the treatment of BV has been unsuccessful to date, likely due to lack of understanding of these complex shifts. Presumably other primates can face similar challenges in which such disorders can elevate the risk for preterm labor and unsuccessful birth (62).

The use of primates as models for human diseases, including those of the reproductive tract, requires a thorough understanding of the normal microbiota of these animals. There are a number reports in the literature concerning this question, but few considered the vaginal tract and its bacterial community in any detail. A wealth of information exists on fecal and the gastrointestinal tract microbiota of the great apes (represented by Chimpanzee and Gorilla), and old world monkeys (represented by Baboon and Macaque), but work on the vaginal microbiota is lacking (37, 66, 119). Studies on our closest relative the Chimpanzee (*Pan troglodytes*), have been directed mainly at the gastrointestinal tract (GI) and the comparisons of wild and captive chimpanzees. Pig Tail Macaques are utilized as standardized clinical safety models for vaginal topical microbiocides but no consideration has been given to the structure of the microbial community and its implications for these treatments.

A small set of reports touch lightly on the subject of vaginal microbiota. For example, Skangalis and others attempted to quantify the bacterial vaginal composition of the baboon using culture dependent methods (104, 105). Their

report revealed Bacteroidetes and Actinobacteria species as the major constituents of the vaginal tract in baboons, followed by Lactobacilli species found in 46% of their subjects. The isolation of lactobacilli from baboons lead to the belief that these animals were suitable for modeling since their microbiota was similar to that of humans. In the case of our closest relative the Chimpanzee (*Pan troglodytes*), a lack of information prevails regarding vaginal microbiota composition. A number of culture dependent reports argue that chimpanzees hold the closest similarities to humans given that their vaginal tract microbial composition is mainly dominated by *Lactobacillus* species as has been previously reported (82).

Effect of immunity on reproductive tract microbiota

Is been postulated that evolution of host immune systems allows for a level of complexity in their associated microbiota (78). The gut microbiota for example, is known to be shaped by the adaptive immune system of the host through immunoglobulin A (IgA); an IgA deficiency alters their gut microbial composition (30, 110). The major histocompatibility complex (MHC) had been associated with scent production in a set of genetically identical mice (63). This scent production was correlated with bacterial metabolites and the effects of these metabolites on mating signaling.

Immunobiology of the baboon reproductive tract

Nonhuman primates have been important models of human disease that are increasingly used in biomedical research because of their similarities to humans. Areas like cardiovascular physiology, transplantation, reproduction and infectious diseases have benefited from research in primates (23). Baboons (*Papio hamadryas*) are phylogenetically close enough to humans that their immune system has significant similarity to that found in humans. Analyses of the baboon reproductive tract immunobiology have shown that the distribution and abundance of immune cells is similar to that reported for the human female revealing a high degree of similarity between immunological profiles of these two primates (24).

In the intestinal tract of many animal species, the microbiota is largely determined by the host's diet. However, in the vaginal tract the effect of diet will only be indirect. As a consequence we can infer that other factors would come into play in determining the composition of the microbiota. The immunobiology of the reproductive tract may play a significant role in determining the composition of the microbiota.

It is worth mentioning that the selective power of the immune system has only been tested against known microbial communities that have been exposed to these selective phenomena throughout the hosts evolutionary history (66). A more comprehensive set of experiments, where the intestinal microbiota is challenged by translocation from one host to another, have been performed in

mice and zebrafish (89). Rawls et al., revealed that after transplantation bacterial community structure resembled its community of origin, but the relative abundance of the lineages changed to resemble the normal gut microbial community composition of the recipient host. Thus, differences in community structure between zebrafish and mice arise in part from distinct selective pressures imposed within the gut habitat of each host. Nonetheless, vertebrate responses to microbial colonization of the gut are ancient: Functional genomic studies disclosed shared host responses to their compositionally distinct microbial communities and distinct microbial species that elicit conserved responses.

Since the early age of microbiology, many different microbial species have been isolated and fully characterized from human gastrointestinal, mouth, skin and vaginal samples. However, during the last decade, molecular ecological studies based on ribosomal RNA (rRNA) sequences have revealed that cultivation dependent studies have vastly underestimated microbial diversity within these ecosystems (7, 35, 125). The majority of these studies reported findings that are mainly focused on health and disease aspects of humans and little has been done for nonhuman primates.

In this thesis, results from molecular methodologies are applied to provide a greater understanding of the microbiota in the vaginal tract of baboons. It establishes clear differences between these two hosts and provides a starting point for a challenge of the theory of host-microbe's co-evolution. In human females species like *Corynebacteria*, *Staphylococci*, *Streptococci*, *E. coli* and

Lactobacillus colonize the vagina soon after birth (55). From puberty to menopause, estrogen increases the concentration of some vaginal microbes such as *Lactobacillus spp*., which in turn becomes the dominant microorganism inhabiting and protecting (not always successfully) the vaginal tract from pathogenic species of bacteria and yeasts. It is worth mentioning that lactobacilli are rarely found in vaginal cultures of post-menopausal women but are commonly found in reproductive age healthy women (15, 18, 39, 85). I report here that in baboons, despite their physiological similarities and phylogenetic proximity to humans, *Lactobacillus spp*., are virtually undetected in females of reproductive age. This represents a major difference between these two primates and raises the question of whether baboons are suitable models for human health and disease. Interestingly human menopause appears to be a phenomenon unique to human among primates. However, this remains controversial based on observational studies of nonhuman primates (4, 46, 47).

Chapter 3 describes the first culture independent study of the baboon vaginal microbiota. A large number of the dominant groups of bacteria in the baboon microbiota are different from those found in humans. Moreover, bacterial derived sequences within the same phylogenetic group clustered separately from the human strains, and were different enough to be classified under different genera. These findings have important implications for understanding the dynamics of host microbe relations in the primate vaginal tract.

INFLUENCE OF THE SURROUNDING ENVIRONMENT

Theoretically external environment could influence microbial species abundance in the vaginal tract. However, researchers have yet to demonstrate variation in species diversity among terrestrial microenvironments and it is understood that studies on microbial diversity in microhabitats are notoriously difficult (53). Many studies give attention to soils (32, 33), which are believed to harbor the majority of the environmental microbes, given the presence of major food availability from some of the organic detritus. However, worldwide soil surveys showed no major differences among large-scale habitats in microbial species richness. Many terrestrial nonhuman primates species (like baboons) are in considerable contact with soil environments, however, there is no current basis to expect that differences in soil environment influence vaginal microbes among primates. Additionally, microbial composition differences between soils and baboon vaginal tracts are substantial; leaving the investigator to infer that community structure is rather influenced by primate physiology. Thus, bacteria colonizing the baboon vagina are responding to differences in the biochemistry of their baboon host and probably have important evolutionary consequences. Differences in the microbiota of different primate species, if better understood, could lead to new discoveries about the physiology and evolution of primates and the reflection of this as a physiology indicator that have has been previously missed.

Co-evolution of host and microbes

Diverse microorganisms and microbial communities are a feature of modern life on the Earth, and have probably played an important role in the evolution of life, as we know it. Microorganisms appeared as early as 4 billion years ago but microbial communities living in association with primates developed much more recently. Humans and baboons shared a common ancestor in the Miocene, about 22 to 25 million years old (43). Bacterial lineages on the other hand, diverged hundreds of million years or more in the past. This is true even for strains of the same species. Results reported in Chapter 2 & 3 indicate that the host's vaginal tract perhaps, sampling microorganism from their surroundings, "selects" from the bacterial diversity normally found in the environment for a particular subset of microbes. The lack of overlap between the microbiota of baboons and humans implies that the major differences in microbiota between them are not due to co-evolution. This selection appears to occur at the host species level, given that we document similarities within baboons in this sample.

Inter-individual microbiota variation has been reported previously in the human gastrointestinal tract (50, 80). However, the vaginal tract microbial community composition is not as strongly influenced by diet or other external factor as the gastrointestinal tract is suggested to be (7, 27, 67, 116). Immune responses to the microbiota have been considered as an important part of commensal-host interactions for the establishment of taxonomic groups colonization of this niche (10). Evidence to support this is still scarce. Thus, more

studies on host physiological response in particular primates are needed to define the nature of these interactions.

In Chapter 2 a cultivation-independent molecular approach, DGGE was implemented to determine the microbial inter-individual variation of a colony of baboons with homogenous environmental conditions (diet and habitat). The homogeneous nature of their environment and the genetically inbred makeup of this captive colony provided good controls for this study of inter-individual variation in microbial communities among baboons. The chapter describes significant variations from one baboon to another. Based on these findings, a hypothesis has arisen suggesting the possibility that when a baboon is born and begins to acquire its vaginal microbiota, a particular group of bacteria, consisting of multiple strains and species to which the animal is exposed, is acquired and maintained but that different individuals harbor somewhat different communities. Individual physiological peculiarities and sexual practices may have a greater impact on defining the microbiota than was previously suspected to occur in the vaginal tract.

Although different types of specimens were taken from the same individual, significantly similar or almost identical DGGE patterns were observed in most cases. Chapter 2 describes these findings and suggests that in field studies, where sample collection can be more challenging than in captive populations, a single type of sample can be taken. The ability to focus on a single sample type will also help by increasing the number of animals that can be studied without compromising the comprehensiveness of the study. Additionally,

one specimen collection permits more intensive DNA sequencing analysis because it will not be necessary to perform this type of analysis on multiple samples from the same animal.

Several interesting aspects of baboon vaginal microbiota were observed when compared to human microbial communities reported in the literature. The research described herein underscores a significance of the marked differences between the two hosts, the suitability of baboons as models for women's heath and disease. Therefore the impact these differences have on baboon translational research. On the other hand, these studies open the door to new aspects of hominid evolution and the integration of anthropology and microbiology.

THE IMPORTANCE OF MICROBIAL COMMUNITIES

Community shifts and their impact on host health

Primates and other animals have a multifaceted relationship with the microbial world. In some cases their encounter can be of a pathogenic nature, but most of the interactions between humans and microorganisms do not result in ailment. Microorganisms in closest contact with mammals are those that commonly reside in or on body sites, including the mouth, skin, gastrointestinal tract, and the urogenital tract. The urogenital tract microbiota is primarily comprised of bacteria, although there are some eukaryotes.

Oral microbiota

Within the oral ecosystem, the development of a community usually involves a succession of populations, typically starting with a few pioneer species and increasing in size and diversity throughout the host life span. Regulatory forces influencing and maintaining stability in the oral cavity include host related, microbial related and external factors (73). In the oral cavity of human newborns, streptococci (e.g. *Streptococcus salivarius* and *S. oralis* among others*)*, species are the pioneer organisms (17, 87). These species will fill the niche and modify the habitat. As a result new populations will colonize and a complex community will develop. As the human host acquires other species (*Actinomyces spp*., and in lower proportions Gram negative bacteria such as *Haemophilus*, *Porphyromonas and Prevotella spp.*) colonize different compartments of the mouth creating higher diversity.

Stability and perturbation

Stability of these communities is based on homeostasis, implying mechanisms of compensation between steady states and perturbation. This balance is maintained by a variety of controls aimed at counteracting the perturbations that would upset the steady state. Perturbations include changes in saliva fluidity, chemistry (e.g. pH) and composition (26, 81). Saliva is a complex mixture that enters the oral cavity by particular ducts located in major salivary glands. Although composed of 99% water, it also contains glycoprotein, proteins, hormones, vitamins, urea and several ions in variable concentrations (26, 81).

Reduction in fluidity ultimately lowers the concentrations of these components and promotes bacterial community shifts that result in undesirable colonization by pathogens or disease-causing residents (*Treponema spp*. and some Gram negative anaerobes) that are normally kept as minor constituents of the mouth microbiota. Also, high carbohydrate intake, particularly sucrose, will lower pH selecting for acidogenic bacterial growth like *Lactobacillus spp*. that will in turn cause disease (69). These bacteria groups are able to rapidly metabolize carbohydrates into acid creating a niche for low pH tolerating bacteria and compromising the integrity of tooth enamel.

Animal models

Several species have been used as animal models for dental disease and oral cavity resident microbiota (5, 81, 84, 113, 123). Studies of the oral cavity microbiota have been performed in nonhuman primates, and such reports revealed an oral microbiota similar to that of humans, and subsequently have shown that these animals as suitable models for human periodontal disease and other oral conditions (81, 84). These studies mainly done in macaques, marmosets and baboons found that the microbiota was dominated by streptococci , *Actinomyces spp*., and obligately anaerobic gram-negative rods. Among these negative rods, dominant species of black-pigmented bacteria, such as *Fusobacterium spp*. and *Alcaligence spp*. have been recovered (8, 76).

Skin microbiota

The skin is colonized by a significant number of organisms that live harmlessly as normal residents on its surface. They are found in highest numbers in those anatomical sites where moisture, temperature and nutrient availability (e.g. sebum), presents a suitable place for bacteria to proliferate and survive. Compared to the large number of microorganisms found in other areas of the body, the skin possesses relatively low concentration of bacteria. Constituents of the microbiota that have been consistently found on the skin are mainly Gram-positive bacteria, such as *Staphylococcus* and *Corynebecterium* (21).

Why are there microbes on our skin?

In contrast to other sites in the human body like the gastrointestinal tract, the role of microbes on the surface of the skin has not yet been determined. There are studies dealing with clinical aspects of microbes in the skin that have shed some light on the composition of the bacterial communities established in coetaneous surfaces but many have failed to address their functions (36, 44, 94). A potential role for these communities is the establishment of a first line of defense against pathogens that transiently visit our skin. For example, *Staphylococcus epidermidis* strains have been found to produce lantibiotics also known as bacteriocins (9, 29, 95). These bacteria also occupy site that could be colonized by pathogens.

Additionally, skin bacteria stimulates innate immune responses of keratinocytes through Toll-like receptor (TLR) signaling (21). This signaling is important in the recognition of pathogens. Thus, normally resident bacteria provide stimulation of the skin's immune system that ultimately may play an important role in defense against harmful pathogens. Although considered a pathogen that can cause life-threatening diseases when the host immunity is compromised or the skin barrier is breached, *Staphylococcus aureus* is also known to produce antimicrobial peptides that deter the colonization of other undesirable microorganisms (45). In healthy human hosts, *S. aureus* is considered to be a normal constituent of the nasal and skin environments. Similar to *S*. *aureus*, *Corynebacterium* spp., *Propionibacterium* spp., Group A Streptococci that are commonly found on the skin surface are known to produce antimicrobial products that help to prevent colonization by pathogens. A clear case of this phenomenon and its influence in the microbiota structure is observed in the case of strains of *Pseudomona aeruginosa* whose products inhibit pathogenic strains of *S*. *aureus* and prevent fungal infections caused by *Candida* spp. (57, 86, 109). The complex interactions reported in the skin microbiota have a beneficial role, much like the ones reported in the gastrointestinal tract.

Gastrointestinal tract microbiota

A complex community of microbes inhabits the mammalian gastrointestinal (GI) tract. For example, in humans it has been estimated that each person possesses a microbiota consisting of 10^{14} organisms, the majority of

which reside in the lower intestinal tract. In the past colonic bacteria were viewed as a collection of free loading "commensal" organisms that took advantage of a ready source of nutrients and provided little input to their hosts. Recently investigators have begun to appreciate that the relationship between the host and its colonic microbiota is an intricate mutualistic symbiosis (7, 68, 77). This microbial ecosystem serves numerous important roles, including protection against pathogens, nutrient processing and stimulation of mucosal cell turn over. Studies in gnotobiotic mice have been particularly informative, illustrating essential roles played by the GI microbiota on host gut development (90, 118). Conversely, a number of diseases have been associated with shifts in the GI microbiota. Cases of inflammatory bowel disease and necrotizing enterocolitis are examples of this phenomenon and had been suspected of having links with microbiota constituents. In addition to bacteria the intestinal microbiota also contains organisms from the Eukaria (e.g., yeast) and Archaea (e.g., methanogens).

Functional aspects of the intestinal microbiota

In return for secure environmental niches, the intestinal microbiota has a number of key functions that contribute to the proper operation of the host gastrointestinal tract. The flow of nutrients between different members of a given ecosystem is influenced by and can ultimately influence the microbiota structure. In the intestinal tract, the bacterial constituents not only have access to a ready supply of nutrients but their metabolites can benefit their hosts. This implies that

for many members of the gut microbiota, their relationship with the host can be considered to be mutualistic. Members of the Firmicutes and Bacteroidetes phyla (GI dominant taxa) for example, ferment non-digestible starch and plant cell wall polysaccharides into short chain fatty acids (72). The major short chain fatty acids are acetate, propionate and butyrate where the latter is the preferred energy source for colonic enterocytes and is believed to contribute to gut homeostasis through modulation of cell function (25).

Aspects of postnatal gut maturation influenced by resident microbes have been described mainly through studies on gnobiotic mice (14). Significant shifts in the intestinal cell surface glycocunjugate (carbohydrates covalently linked to other species) repertoire have been linked to the presence of species of *Bacteroides* in the intestinal tract (51, 124). As discussed earlier in this chapter, another important aspect of the host-microbiota interaction involves the immune system. The microbiota in this ecosystem have been reported to stimulate beneficial as well as deleterious host immune responses. Less is known about immune system effects on the structuring of the intestinal microbiota. However, early work reveals that in mice, the lack of immune system components such as IgA alters the microbiota where a favored expansion of segmented filamentous bacteria is observed (111). Details of the mechanism of such phenomena are yet to be described.

It is clear that the presence of bacterial species in the GI tract plays a critical role in host development, homeostasis and ultimately good health state. It is also clear that the concept of a “normal” microbiota is not limited to merely a

random collection of “commensal” organisms that take advantage of easy access to a nutrient rich environment. Instead, it can be considered a dynamic interplay between host and microbes in which indigenous microorganisms, host mucosal epithelium and immune system elements exist in equilibrium. This equilibrium represents a stable interdependence between the various components that contribute to the survival of each individual element.

The vaginal microbiota

Starting from first hours after delivery of an infant from the sterile uterine environment, the interaction of the newborn with microorganisms begins. The main portal of entry for microbes is skin and mucosal surfaces of the gastrointestinal, respiratory and urogenital tracts. Physiologically occurring interactions with bacteria leads to colonization of epithelial surfaces and this co-existence is usually harmonious and beneficial for the host.

In humans, under normal physiological conditions, the vaginal tract primarily harbors lactobacilli that colonize the vaginal epithelium and confer resistance to other microorganisms, such as yeast that cause vaginitis. Albeit only a few *Lactobacillus* species constitute the vaginal microbiota, significant species- and strain-specific differences do occur and these differences account for a wide variability in the intrinsic capability of the *Lactobacillus* strains to maintain the vaginal ecosystem. Hence, among a substantial proportion of women, the picture of lactobacilli-driven mutualism is actually less ideal than one would assume. As the vagina is incessantly subjected to cyclic changes as well

as behavioral exposures that may challenge the perpetuation of the *Lactobacillus* populations, the intrinsic stability of the resident microbiota is paramount to a woman's health.

Dominant species in the vaginal microbiota

Loss of the hydrogen peroxide producing lactobacilli, accompanied by massive Gram-negative anaerobes overgrowth, is observed with bacterial vaginosis (35, 125). Molecular studies of the bacterial vaginosis microflora have recently revealed tremendous species variability, further documenting the complex polymicrobial nature of this condition (115). Emerging issues include the predominance of *Gardnerella vaginalis* in BV patients, the associated abundance of *Atopobium vaginae* as a rather specific marker of therapy failure and disease persistence or recurrence, and the apperance of an adherent, metronidazole-resistant biofilm consisting of the latter two species (31).

The first basic element to be considered in the microecology of the vaginal microbiota is the vaginal epithelium. The vagina of humans and some non-human primates is lined by a stratified, squamous nonkeratinized epithelium that proliferates and thickens in response to elevated estrogen levels. Secretions arise from Bartholin’s and Skene’s glands, cervical mucus and endometrial and fallopian sites. Vaginal transudation is controlled by estrogen levels and is reduced after midcycle when estrogen levels decrease. The secretions consist of 90–95% water, inorganic and organic salts, urea, carbohydrates, mucins, fatty

acids, albumins, inmunoglobulins, iron chelators, lysozyme and other macromolecules, leukocyte and epithelial debris (11, 64, 79, 91).

The second element comprising the vaginal ecosystem is the microbiota. Doderlein published the first extensive study of human vaginal microbiota in 1892. Doderlein and his contemporaries considered the vaginal microbiota to be homogenous, consisting only of Gram-positive bacilli; these Doderlein's bacilli are now known to be a member of the genus *Lactobacillus* (91). This old concept that the vagina is colonized almost solely by lactobacilli has been modified by researchers who have used contemporary methods and have found that the microbiota of asymptomatic women consists typically of a diversity of anaerobic and aerobic microorganisms (79). However, lactobacilli are the most prevalent and are often numerically dominant microorganism, at 10^7 to 10^8 of vaginal fluid in healthy premenopausal women (2). In earlier studies, the predominant species isolated were *Lactobacillus acidophilus* and *L. fermentum*, with lower levels of *L. plantarum*, *L. brevis*, *L. jensenii*, *L. casei*, *L. delbrueckii* and *L. salivarius*. Additionally, using culture independent methods, scientist found that the identification of *L. acidophilus* was erroneous and that *L. crispatus*, *L. fermentum*, *L. jensenii* and *L. gasseri* were the predominant vaginal species (13, 42).

The third aspect of the vaginal microecology is the complexity of host-microbe interactions and interactions among microbial species. These interactions may be antagonistic or synergistic. For example, lactobacilli use two mechanisms to interfere with pathogens. One is adherence to the mucus, forming a barrier, which prevents colonization by pathogens. Two, the production

of antimicrobial compounds such as hydrogen peroxide, lactic acid, bacteriocin-like substances and possibly, biosurfactants discourage colonization by some pathogens.

Low vaginal pH and production of lactic acid

The low vaginal pH of the human vagina appears to be an important mechanism for controlling in controlling the composition of microbiota. Because of the finding that the vaginas of reproductive-aged women typically have a pH of approximately 4–4.5, it has commonly been believed that this degree of acidity strictly limited the microbiota to acidophilic or aciduric species such as *Lactobacillus* spp. It is likely that pH may have more subtle effects than simply to provide an unfavorable environment for certain species of microorganisms. Many organisms have a broad range of pH values at which they are able to replicate, but certain enzyme systems may be affected by changes in pH. The lactic acid and other fatty acids produced by *Lactobacillus* metabolism may contribute to vaginal acidity, but this is not necessarily the primary source of low vaginal pH; fatty acids, including lactic acid, produced by vaginal epithelial cells and released into the secretions are probably a more important source (88). However, in vitro studies have shown that acidification by lactobacilli growth can inhibit the proliferation of pathogenic microorganisms, such as *Candida albicans* (60), *Escherichia coli* (93), *G. vaginalis*, *Mobiluncus spp*. and other bacteria cultured from vaginal specimens obtained from women with bacterial vaginosis (106).

As discussed above, lactobacilli are thought to play an important role in protecting the human host from genital infections (12, 92). However, in previous reports, lactobacilli were not the predominant bacteria in the vaginal tracts of many laboratory animals, including mice, rats, hamsters, rabbits, dogs and goats (83). However, it is not clear whether such laboratory animals are suitable as experimental models for studying the role of the vaginal microbiota of human beings. In an another report, quantitative analysis of the vaginal microbiota of conventionally reared mice, rats, hamsters, rabbits, dogs were done using the same analytical method (83). The most predominant bacteria in the vagina of laboratory animals were: streptococci in mice; Gram-negative rods (GNR), streptococci, and members of the family Bacteroidaceae in rats; GNR, Bacteroidaceae, and gram-positive anaerobic cocci (GPAC) in hamsters; and Bacteroidaceae in dogs. Thus, lactobacilli are not predominant in the vagina of these laboratory animals, and they are therefore not suitable subjects for studying the role of lactobacilli in the vagina of human beings.

Primate species are taxonomically closer to human beings and would be expected to serve as better models. However, as I demonstrate in this thesis, the vaginal microbiota of baboons is also different from that of humans.

REFERENCES

1. **Anderson, D. M.** 2008. The nonhuman primate as a model for biomedical research. Humana Press Inc., Totowa, NJ.

2. **Andreu, A., A. E. Stapleton, C. L. Fennell, S. L. Hillier, and W. E. Stamm.** 1995. Hemagglutination, adherence, and surface properties of vaginal *Lactobacillus* species. J Infect Dis **171:**1237-43.

3. **Andrieux, C., J. M. Membre, C. Cayuela, and J. M. Antoine.** 2002. Metabolic characteristics of the faecal microflora in humans from three age groups. Scand J Gastroenterol **37:**792-8.

4. **Atsalis, S., and S. Margulis.** 2008. Primate reproductive aging: from lemurs to humans. Interdiscip Top Gerontol **36:**186-94.

5. **Avery, B. E., and D. M. Simpson.** 1973. The baboon as a model system for the study of periodontal disease: clinical and light microscopic observations. J Periodontol **44:**675-86.

6. **Avila, M., D. M. Ojcius, and O. Yilmaz.** 2009. The oral microbiota: living with a permanent guest. DNA Cell Biol **28:**405-11.

7. **Backhed, F., R. E. Ley, J. L. Sonnenburg, D. A. Peterson, and J. I. Gordon.** 2005. Host-bacterial mutualism in the human intestine. Science **307:**1915-20.

8. **Beem, J. E., C. G. Hurley, I. Magnusson, W. P. McArthur, and W. B. Clark.** 1991. Subgingival microbiota in squirrel monkeys with naturally occurring periodontal diseases. Infect Immun **59:**4034-41.

9. **Bierbaum, G., F. Gotz, A. Peschel, T. Kupke, M. van de Kamp, and H. G. Sahl.** 1996. The biosynthesis of the lantibiotics epidermin, gallidermin, Pep5 and epilancin K7. Antonie Van Leeuwenhoek **69:**119-127.

10. **Blum, S., S. Alvarez, D. Haller, P. Perez, and E. J. Schiffrin.** 1999. Intestinal microflora and the interaction with immunocompetent cells. Antonie Van Leeuwenhoek **76:**199-205.

11. **Boris, S., and C. Barbes.** 2000. Role played by lactobacilli in controlling the population of vaginal pathogens. Microbes Infect **2:**543-6.

12. **Boris, S., J. E. Suarez, F. Vazquez, and C. Barbes.** 1998. Adherence of human vaginal lactobacilli to vaginal epithelial cells and interaction with uropathogens. Infect Immun **66:**1985-9.

13. **Brown, C. J., M. Wong, C. C. Davis, A. Kanti, X. Zhou, and L. J. Forney.** 2007. Preliminary characterization of the normal microbiota of the human vulva using cultivation-independent methods. J Med Microbiol **56:**271-6.

14. **Bry, L., P. G. Falk, T. Midtvedt, and J. I. Gordon.** 1996. A model of host-microbial interactions in an open mammalian ecosystem. Science **273:**1380-3.

15. **Burton, J. P., and G. Reid.** 2002. Evaluation of the bacterial vaginal flora of 20 postmenopausal women by direct (Nugent score) and molecular (polymerase chain reaction and denaturing gradient gel electrophoresis) techniques. J Infect Dis **186:**1770-80.

16. **Caccone, A., and J. R. Powell.** 1989. DNA Divergence among hominoids. Evolution **43:**925-942.

17. **Carlsson, J., H. Grahnen, G. Jonsson, and S. Wikner.** 1970. Early establishment of Streptococcus salivarius in the mouth of infants. J Dent Res **49:**415-8.

18. **Cauci, S., S. Driussi, D. De Santo, P. Penacchioni, T. Iannicelli, P. Lanzafame, F. De Seta, F. Quadrifoglio, D. de Aloysio, and S. Guaschino.** 2002. Prevalence of bacterial vaginosis and vaginal flora changes in peri- and postmenopausal women. J Clin Microbiol **40:**2147-52.

19. **Chavez, A. O., J. C. Lopez-Alvarenga, M. E. Tejero, C. Triplitt, R. A. Bastarrachea, A. Sriwijitkamol, P. Tantiwong, V. S. Voruganti, N. Musi, A. G. Comuzzie, R. A. DeFronzo, and F. Folli.** 2008. Physiological and molecular determinants of insulin action in the baboon. Diabetes **57:**899-908.

20. **Christersson, L. A., J. J. Zambon, and R. J. Genco.** 1991. Dental bacterial plaques. Nature and role in periodontal disease. J Clin Periodontol **18:**441-6.

21. **Cogen, A. L., V. Nizet, and R. L. Gallo.** 2008. Skin microbiota: a source of disease or defence? Br J Dermatol **158:**442-55.

22. **Coolen, M. J., E. Post, C. C. Davis, and L. J. Forney.** 2005. Characterization of microbial communities found in the human vagina by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. Appl Environ Microbiol **71:**8729-37.

23. **D'Hooghe, T. M., C. S. Bambra, B. M. Raeymaekers, and P. R. Koninckx.** 1996. Increased prevalence and recurrence of retrograde menstruation in baboons with spontaneous endometriosis. Hum Reprod **11:**2022-5.

24. **D'Hooghe, T. M., L. Xiao, and J. A. Hill.** 2001. Cytokine profiles in autologous peritoneal fluid and peripheral blood of women with deep and superficial endometriosis. Arch Gynecol Obstet **265:**40-4.

25. **Daly, K., M. A. Cuff, F. Fung, and S. P. Shirazi-Beechey.** 2005. The importance of colonic butyrate transport to the regulation of genes associated with colonic tissue homoeostasis. Biochem Soc Trans **33:**733-5.

26. **Dawes, C.** 1970. Effects of diet on salivary secretion and composition. J Dent Res **49:**1263-73.

27. **DiBaise, J. K., H. Zhang, M. D. Crowell, R. Krajmalnik-Brown, G. A. Decker, and B. E. Rittmann.** 2008. Gut microbiota and its possible relationship with obesity. Mayo Clin Proc **83:**460-9.

28. **Donders, G. G.** 2007. Definition and classification of abnormal vaginal flora. Best Pract Res Clin Obstet Gynaecol **21:**355-73.

29. **Ekkelenkamp, M. B., M. Hanssen, S. T. Danny Hsu, A. de Jong, D. Milatovic, J. Verhoef, and N. A. van Nuland.** 2005. Isolation and structural characterization of epilancin 15X, a novel lantibiotic from a clinical strain of *Staphylococcus epidermidis*. FEBS Lett **579:**1917-22.

30. **Fagarasan, S., M. Muramatsu, K. Suzuki, H. Nagaoka, H. Hiai, and T. Honjo.** 2002. Critical roles of activation-induced cytidine deaminase in the homeostasis of gut flora. Science **298:**1424-7.

31. **Ferris, M. J., A. Masztal, K. E. Aldridge, J. D. Fortenberry, P. L. Fidel, Jr., and D. H. Martin.** 2004. Association of Atopobium vaginae, a recently described metronidazole resistant anaerobe, with bacterial vaginosis. BMC Infect Dis **4:**5.

32. **Fierer, N., M. A. Bradford, and R. B. Jackson.** 2007. Toward an ecological classification of soil bacteria. Ecology **88:**1354-64.

33. **Fierer, N., J. A. Jackson, R. Vilgalys, and R. B. Jackson.** 2005. Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. Appl Environ Microbiol **71:**4117-20.

34. **Flint, H. J., E. A. Bayer, M. T. Rincon, R. Lamed, and B. A. White.** 2008. Polysaccharide utilization by gut bacteria: potential for new insights from genomic analysis. Nat Rev Microbiol **6:**121-31.

35. **Forney, L. J., J. A. Foster, and W. Ledger.** 2006. The vaginal flora of healthy women is not always dominated by Lactobacillus species. J Infect Dis **194:**1468-9; author reply 1469-70.

36. **Fredricks, D. N.** 2001. Microbial ecology of human skin in health and disease. J Investig Dermatol Symp Proc **6:**167-9.

37. **Frey, J. C., J. M. Rothman, A. N. Pell, J. B. Nizeyi, M. R. Cranfield, and E. R. Angert.** 2006. Fecal bacterial diversity in a wild gorilla. Appl Environ Microbiol **72:**3788-92.

38. **Fujisawa, T., Y. Benno, and T. Mitsuoka.** 1992. Effect of menstrual cycle and different age on vaginal microflora of healthy women. Bifidobacteria Microflora**:**33-38.

39. **Galhardo, C. L., J. M. Soares, Jr., R. S. Simoes, M. A. Haidar, G. Rodrigues de Lima, and E. C. Baracat.** 2006. Estrogen effects on the vaginal pH, flora and cytology in late postmenopause after a long period without hormone therapy. Clin Exp Obstet Gynecol **33:**85-9.

40. **Garber, P. A.** 2009. Nonhuman primate models of women's health. Am J Primatol **71:**713-4.

41. **Gardner, H. L., and C. D. Dukes.** 1955. *Haemophilus vaginalis* vaginitis: a newly defined specific infection previously classified non-specific vaginitis. Am J Obstet Gynecol **69:**962-76.

42. **Giorgi, A., S. Torriani, F. Dellaglio, G. Bo, E. Stola, and L. Bernuzzi.** 1987. Identification of vaginal lactobacilli from asymptomatic women. Microbiologica **10:**377-84.

43. **Glazko, G. V., and M. Nei.** 2003. Estimation of divergence times for major lineages of primate species. Mol Biol Evol **20:**424-34.

44. **Hadaway, L. C.** 2003. Skin flora and infection. J Infus Nurs **26:**44-8.

45. **Hale, E. M., and R. D. Hinsdill.** 1975. Biological activity of staphylococcin 162: bacteriocin from *Staphylococcus aureus*. Antimicrob Agents Chemother **7:**74-81.

46. **Hawkes, K.** 2003. Grandmothers and the evolution of human longevity. Am J Hum Biol **15:**380-400.

47. **Hawkes, K.** 2004. Human longevity: the grandmother effect. Nature **428:**128-9.

48. **Heath, P. T., and A. Schuchat.** 2007. Perinatal group B streptococcal disease. Best Pract Res Clin Obstet Gynaecol **21:**411-24.

49. **Hill, G. B.** 1993. The microbiology of bacterial vaginosis. Am J Obstet Gynecol **169:**450-4.

50. **Holdeman, L. V., I. J. Good, and W. E. Moore.** 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol **31:**359-75.

51. **Hooper, L. V., M. H. Wong, A. Thelin, L. Hansson, P. G. Falk, and J. I. Gordon.** 2001. Molecular analysis of commensal host-microbial relationships in the intestine. Science **291:**881-4.

52. **Hooton, T. M., S. Hillier, C. Johnson, P. L. Roberts, and W. E. Stamm.** 1991. *Escherichia coli* bacteriuria and contraceptive method. Jama **265:**64-9.

53. **Hughes, J. B., J. J. Hellmann, T. H. Ricketts, and B. J. Bohannan.** 2001. Counting the uncountable: statistical approaches to estimating microbial diversity. Appl Environ Microbiol **67:**4399-406.

54. **Hyman, R. W., M. Fukushima, L. Diamond, J. Kumm, L. C. Giudice, and R. W. Davis.** 2005. Microbes on the human vaginal epithelium. Proc Natl Acad Sci U S A **102:**7952-7.

55. **Jarvis, W. R.** 1996. The epidemiology of colonization. Infect Control Hosp Epidemiol **17:**47-52.

56. **Johansson, M., and N. Y. Lycke.** 2003. Immunology of the human genital tract. Curr Opin Infect Dis **16:**43-9.

57. **Kerr, J. R.** 1994. Suppression of fungal growth exhibited by *Pseudomonas aeruginosa*. J Clin Microbiol **32:**525-7.

58. **Kiss, H., B. Kogler, L. Petricevic, I. Sauerzapf, S. Klayraung, K. Domig, H. Viernstein, and W. Kneifel.** 2007. Vaginal *Lactobacillus* microbiota of healthy women in the late first trimester of pregnancy. Bjog **114:**1402-7.

59. **Koumans, E. H., and J. S. Kendrick.** 2001. Preventing adverse sequelae of bacterial vaginosis: a public health program and research agenda. Sex Transm Dis **28:**292-7.

60. **Krasner, R. I., G. Young, and P. L. Yudkofsky.** 1956. Interactions of oral strains of Candida albicans and lactobacilli. J Bacteriol **72:**525-9.

61. **Lacreuse, A., and J. G. Herndon.** 2009. Nonhuma primate models for cognitive aging., p. 29-258. *In* J. J. Bizon and J. Woods (ed.), Animal models of human cognitive aging. Humana Press Inc., Totawa, NJ.

62. **Lamont, R. F., and S. R. Sawant.** 2005. Infection in the prediction and antibiotics in the prevention of spontaneous preterm labour and preterm birth. Minerva Ginecol **57:**423-33.

63. **Lanyon, C. V., S. P. Rushton, G. O'Donnell A, M. Goodfellow, A. C. Ward, M. Petrie, S. P. Jensen, L. Morris Gosling, and D. J. Penn.** 2007. Murine scent mark microbial communities are genetically determined. FEMS Microbiol Ecol **59:**576-83.

64. **Larsen, B.** 1993. Vaginal flora in health and disease. Clin Obstet Gynecol **36:**107-21.

65. **Leitich, H., and H. Kiss.** 2007. Asymptomatic bacterial vaginosis and intermediate flora as risk factors for adverse pregnancy outcome. Best Pract Res Clin Obstet Gynaecol **21:**375-90.

66. **Ley, R. E., C. A. Lozupone, M. Hamady, R. Knight, and J. I. Gordon.** 2008. Worlds within worlds: evolution of the vertebrate gut microbiota. Nat Rev Microbiol **6:**776-88.

67. **Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon.** 2006. Microbial ecology: human gut microbes associated with obesity. Nature **444:**1022-3.

68. **Lievin-Le Moal, V., and A. L. Servin.** 2006. The front line of enteric host defense against unwelcome intrusion of harmful microorganisms: mucins, antimicrobial peptides, and microbiota. Clin Microbiol Rev **19:**315-37.

69. **Loesche, W. J.** 1986. Role of *Streptococcus mutans* in human dental decay. Microbiol Rev **50:**353-80.

70. **Magness, C. L., P. C. Fellin, M. J. Thomas, M. J. Korth, M. B. Agy, S. C. Proll, M. Fitzgibbon, C. A. Scherer, D. G. Miner, M. G. Katze, and S. P. Iadonato.** 2005. Analysis of the *Macaca mulatta* transcriptome and the sequence divergence between *Macaca* and human. Genome Biol **6:**R60.

71. **Mahoney, C. J.** 1970. Induction of ovulation in *Macaca irus* with clomiphene citrate and gonadotrophic hormones. J Reprod Fertil **23:**529-31.

72. **Mahowald, M. A., F. E. Rey, H. Seedorf, P. J. Turnbaugh, R. S. Fulton, A. Wollam, N. Shah, C. Wang, V. Magrini, R. K. Wilson, B. L. Cantarel, P. M. Coutinho, B. Henrissat, L. W. Crock, A. Russell, N. C. Verberkmoes, R. L. Hettich, and J. I. Gordon.** 2009. Characterizing a model human gut microbiota composed of members of its two dominant bacterial phyla. Proc Natl Acad Sci U S A **106:**5859-64.

73. **Marsh, P. D., and M. Martin (ed.).** 1992. Oral Microbiology, 3rd ed. Chapman & Hall, Ltd., London, United Kingdom.

74. **Martens, E. C., R. Roth, J. E. Heuser, and J. I. Gordon.** 2009. Coordinate regulation of glycan degradation and polysaccharide capsule biosynthesis by a prominent human gut symbiont. J Biol Chem **284:**18445-57.

75. **Masfari, A. N., B. I. Duerden, and G. R. Kinghorn.** 1986. Quantitative studies of vaginal bacteria. Genitourin Med **62:**256-63.

76. **Mashimo, P. A., S. A. Ellison, and J. Slots.** 1979. Microbial composition of monkey dental plaque (*Macaca arctoides* and *Macaca fascicularis*). Scand J Dent Res **87:**24-31.

77. **McCracken, V. J., and R. G. Lorenz.** 2001. The gastrointestinal ecosystem: a precarious alliance among epithelium, immunity and microbiota. Cell Microbiol **3:**1-11.

78. **McFall-Ngai, M.** 2007. Adaptive immunity: care for the community. Nature **445:**153.

79. **McGroarty, J. A.** 1993. Probiotic use of lactobacilli in the human female urogenital tract. FEMS Immunol Med Microbiol **6:**251-64.

80. **Moore, W. E., and L. V. Holdeman.** 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol **27:**961-79.

81. **Navia, J. M.** 1977. Animal model ins dental research. The University of Alabama Press, Birmingham.

82. **Noguchi, K., K. Tsukumi, T. Udono, and T. Urano.** 2004. Normal vaginal flora in chimpanzees (*Pan troglodytes*): qualitative and quantitative study. Comp Med **54:**705-12.

83. **Noguchi, K., K. Tsukumi, and T. Urano.** 2003. Qualitative and quantitative differences in normal vaginal flora of conventionally reared mice, rats, hamsters, rabbits, and dogs. Comp Med **53:**404-12.

84. **Nolte, W. A.** 1982. Oral Microbiology: with basic microbiology and inmunology, 4th ed. Th C.V. Mosby Co., St. Louis, MO.

85. **Pabich, W. L., S. D. Fihn, W. E. Stamm, D. Scholes, E. J. Boyko, and K. Gupta.** 2003. Prevalence and determinants of vaginal flora alterations in postmenopausal women. J Infect Dis **188:**1054-8.

86. **Padilla, C., O. Lobos, E. Hubert, F. Poblete, A. Navarro, and L. Nunez.** 2006. In vitro antibacterial activity of the peptide PsVP-10 against *Streptococcus mutans* and *Streptococcus sobrinus* with and without glycocalyx. Int J Antimicrob Agents **27:**212-6.

87. **Pearce, C., G. H. Bowden, M. Evans, S. P. Fitzsimmons, J. Johnson, M. J. Sheridan, R. Wientzen, and M. F. Cole.** 1995. Identification of pioneer viridans streptococci in the oral cavity of human neonates. J Med Microbiol **42:**67-72.

88. **Preti, G., and G. R. Huggins.** 1975. Cyclical changes in volatile acidic metabolites of human vaginal secretions and their relation to ovulation. Journal of Chemical Ecology **1:**361-S376.

89. **Rawls, J. F., M. A. Mahowald, R. E. Ley, and J. I. Gordon.** 2006. Reciprocal gut microbiota transplants from zebrafish and mice to germ-free recipients reveal host habitat selection. Cell **127:**423-33.

90. **Rawls, J. F., B. S. Samuel, and J. I. Gordon.** 2004. Gnotobiotic zebrafish reveal evolutionarily conserved responses to the gut microbiota. Proc Natl Acad Sci U S A **101:**4596-601.

91. **Redondo-Lopez, V., R. L. Cook, and J. D. Sobel.** 1990. Emerging role of lactobacilli in the control and maintenance of the vaginal bacterial microflora. Rev Infect Dis **12:**856-72.

92. **Reid, G., and J. Burton.** 2002. Use of *Lactobacillus* to prevent infection by pathogenic bacteria. Microbes Infect **4:**319-24.

93. **Reid, G., J. A. McGroarty, R. Angotti, and R. L. Cook.** 1988. *Lactobacillus* inhibitor production against *Escherichia coli* and coaggregation ability with uropathogens. Can J Microbiol **34:**344-51.

94. **Roth, R. R., and W. D. James.** 1989. Microbiology of the skin: resident flora, ecology, infection. J Am Acad Dermatol **20:**367-90.

95. **Sahl, H. G.** 1994. Staphylococcin 1580 is identical to the lantibiotic epidermin: implications for the nature of bacteriocins from gram-positive bacteria. Appl Environ Microbiol **60:**752-5.

96. **Salyers, A. A., J. R. Vercellotti, S. E. West, and T. D. Wilkins.** 1977. Fermentation of mucin and plant polysaccharides by strains of *Bacteroides* from the human colon. Appl Environ Microbiol **33:**319-22.

97. **Sautter, R. L., and W. J. Brown.** 1980. Sequential vaginal cultures from normal young women. J Clin Microbiol **11:**479-84.

98. **Schwebke, J. R.** 2005. Trichomoniasis in adolescents: a marker for the lack of a public health response to the epidemic of sexually transmitted diseases in the United States. J Infect Dis **192:**2036-8.

99. **Schwebke, J. R., and R. Desmond.** 2005. Risk factors for bacterial vaginosis in women at high risk for sexually transmitted diseases. Sex Transm Dis **32:**654-8.

100. **Scinicariello, F., L. Jayashankar, and R. Attanasio.** 2002. Baboon immunoglobulin variable region heavy chains: identification of genes homologous to members of the human IGHV1-IGHV7 subgroups. Immunogenetics **53:**815-20.

101. **Sibal, L. R., and K. J. Samson.** 2001. Nonhuman primates: a critical role in current disease research. Ilar J **42:**74-84.

102. **Sibley, C. G., and J. E. Ahlquist.** 1987. DNA hybridization evidence of hominoid phylogeny: results from an expanded data set. J Mol Evol **26:**99-121.

103. **Sibley, C. G., and J. E. Ahlquist.** 1984. The phylogeny of the hominoid primates, as indicated by DNA-DNA hybridization. J Mol Evol **20:**2-15.

104. **Skangalis, M., C. J. Mahoney, and W. M. O'Leary.** 1982. Microbial presence in the uterine cavity as affected by varieties of intrauterine contraceptive devices. Fertil Steril **37:**263-9.

105. **Skangalis, M., C. E. Swenson, C. J. Mahoney, and W. M. O'Leary.** 1979. The normal microbial flora of the baboon vagina. J Med Primatol **8:**289-97.

106. **Skarin, A., and J. Sylwan.** 1986. Vaginal lactobacilli inhibiting growth of Gardnerella vaginalis, Mobiluncus and other bacterial species cultured from vaginal content of women with bacterial vaginosis. Acta Pathol Microbiol Immunol Scand B **94:**399-403.

107. **Steinetz, B. G., C. Randolph, and C. J. Mahoney.** 1992. Serum concentrations of relaxin, chorionic gonadotropin, estradiol-17 beta, and progesterone during the reproductive cycle of the chimpanzee (Pan troglodytes). Endocrinology **130:**3601-7.

108. **Stevens, V. C.** 1997. Some reproductive studies in the baboon. Hum Reprod Update **3:**533-40.

109. **Sutherland, R., R. J. Boon, K. E. Griffin, P. J. Masters, B. Slocombe, and A. R. White.** 1985. Antibacterial activity of mupirocin (pseudomonic acid), a new antibiotic for topical use. Antimicrob Agents Chemother **27:**495-8.

110. **Suzuki, K., S. A. Ha, M. Tsuji, and S. Fagarasan.** 2007. Intestinal IgA synthesis: a primitive form of adaptive immunity that regulates microbial communities in the gut. Semin Immunol **19:**127-35.

111. **Suzuki, K., B. Meek, Y. Doi, M. Muramatsu, T. Chiba, T. Honjo, and S. Fagarasan.** 2004. Aberrant expansion of segmented filamentous bacteria in IgA-deficient gut. Proc Natl Acad Sci U S A **101:**1981-6.

112. **Taha, T. E., D. R. Hoover, G. A. Dallabetta, N. I. Kumwenda, L. A. Mtimavalye, L. P. Yang, G. N. Liomba, R. L. Broadhead, J. D. Chiphangwi, and P. G. Miotti.** 1998. Bacterial vaginosis and disturbances of vaginal flora: association with increased acquisition of HIV. Aids **12:**1699-706.

113. **Tanzer, J. M.** 1981. Animal models in cariology. Information Retrieval Inc., Washington, D.C.

114. **Thies, F. L., W. Konig, and B. Konig.** 2007. Rapid characterization of the normal and disturbed vaginal microbiota by application of 16S rRNA gene terminal RFLP fingerprinting. J Med Microbiol **56:**755-61.

115. **Thorsen, P., I. P. Jensen, B. Jeune, N. Ebbesen, M. Arpi, A. Bremmelgaard, and B. R. Moller.** 1998. Few microorganisms associated with bacterial vaginosis may constitute the pathologic core: a population-based microbiologic study among 3596 pregnant women. Am J Obstet Gynecol **178:**580-7.

116. **Turnbaugh, P. J., F. Backhed, L. Fulton, and J. I. Gordon.** 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe **3:**213-23.

117. **Turnbaugh, P. J., R. E. Ley, M. Hamady, C. M. Fraser-Liggett, R. Knight, and J. I. Gordon.** 2007. The human microbiome project. Nature **449:**804-10.

118. **Turnbaugh, P. J., R. E. Ley, M. A. Mahowald, V. Magrini, E. R. Mardis, and J. I. Gordon.** 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. Nature **444:**1027-31.

119. **Uenishi, G., S. Fujita, G. Ohashi, A. Kato, S. Yamauchi, T. Matsuzawa, and K. Ushida.** 2007. Molecular analyses of the intestinal microbiota of chimpanzees in the wild and in captivity. Am J Primatol **69:**367-76.

120. **VandeBerg, J. L., and S. Williams-Blangero.** 1997. Advantages and limitations of nonhuman primates as animal models in genetic research on complex diseases. J Med Primatol **26:**113-9.

121. **Verstraelen, H., and A. C. Senok.** 2005. Vaginal lactobacilli, probiotics, and IVF. Reprod Biomed Online **11:**674-5.

122. **Wilks, J. W.** 1979. A procedure for evaluating luteolytic agents in primates. Adv Exp Med Biol **112:**757-66.

123. **Wolff, L. F., M. J. Krupp, and W. F. Liljemark.** 1985. Microbial changes associated with advancing periodontitis in STR/N mice. J Periodontal Res **20:**378-85.

124. **Xu, J., M. K. Bjursell, J. Himrod, S. Deng, L. K. Carmichael, H. C. Chiang, L. V. Hooper, and J. I. Gordon.** 2003. A genomic view of the human-*Bacteroides thetaiotaomicron* symbiosis. Science **299:**2074-6.

125. **Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney.** 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology **150:**2565-73.

CHAPTER 2

SIGNIFICANT INTER-INDIVIDUAL VARIATION IN THE VAGINAL MICROBIOTA OF BABOONS LIVING IN THE SAME FACILITY

INTRODUCTION

The microbiologists who undertook the first systematic study of the human intestinal microbiota in the 1960s and 1970s were surprised to find that there appeared to be considerable person-to-person variation in microbial composition (9, 14). The major metabolic groups were conserved between individuals; it was the species that varied. Since hosts of the same species appeared to have the same gastrointestinal physiology, this phenomenon seemed counterintuitive. It was tempting to attribute this variation to artifacts introduced by cultivation method used at the time for evaluation of microbiota composition.

Recently, scientists have begun to apply cultivation-independent molecular techniques to evaluating the human intestinal and vaginal microbiota (5, 7, 10, 11, 22). Analysis of the 16S rDNA sequences from both locations confirmed that there was considerable individual-to-individual variation. Though it might be argued that there are artifacts associated with this type of analysis, the biases are different from those associated with cultivation-based methods, and the agreement on inter-individual variation strengthens the conclusion that such variation actually occurs. In the case of human populations, individual variation could be attributable to differences in diet; environment and the

widespread use of antibiotics (6, 19-21). Even in the case of the vaginal tract, where effects of diet and environment would be more indirect than in the intestinal tract, such variations could lead to differences in hormones and the composition of secretions.

In connection with a study designed to evaluate baboons as an animal model for the microbial component of the human reproductive system, we had access to vaginal samples from 35 female baboons that were housed in a research facility in the Southwest National Primate Center in Houston, Texas. These animals were all fed a comparable diet, housed under the same conditions, were highly interrelated and were seldom or never given antibiotics. This presented a good opportunity to assess animal-to-animal variation in the microbiota of a non-human primate. Baboons are widely used as animal models for reproductive studies, testing of contraceptives and such urogenital tract conditions as endometriosis, given that hormone cycles and general physiology of the urogenital tract resemble humans so closely (8, 18).

Since the number of animals was too large to make 16S rRNA gene sequence analysis feasible, we decided to use DGGE as an indicator of microbial community variation between animals. Our approach entails the use of three types of samples from each animal: a swab to sample loosely adherent bacteria, a gentle scrape to sample more adherent bacteria and a lavage sample to collect an approximate representation of the total bacterial population. Testing different types of samples was important because some of the variation seen in the human studies could have been due to differences in the types of samples

evaluated. For instance, in a fecal sample, differences in the water content of intestinal composition can lead to differences in the amount of adherent versus luminal bacteria that would appear as individual-to-individual variation (13).

Here we report remarkable inter-individual variation of a homogeneous population of baboons by the application of DGGE as a rapid means to obtaining a molecular census of their vaginal microbial content. This method allowed us to qualitatively analyze 105 samples from 35 baboons of different ages and menstrual state and establish microbiota variations from individual-to-individual.

MATERIALS AND METHODS

Sample collection

Thirty-five sexually mature female baboons (*Papio hamadryas*) utilized in this study were housed at the Southwest National Primates Research Center (SNPRC) in San Antonio, TX. Vaginal tract sample collection was performed following approved Institutional Animal Care and Use Committee (IACUC) protocols. All primates were fed a diet of Purina monkey chow (no less than 5% protein), fruit and water. No animal received antibiotic treatment during the several months prior to our sampling. Primate manipulation for sampling was carried out strictly after animals were injected intra-muscularly with ketamine hydrochloride at a dosage of 10-15 mg/kg bodyweight.

Three samples were collected from each baboon in this study to recover DNA from vaginal microbes present. Prior to any vaginal tract insertions for sample collection all exterior regions were wiped clean with isopropanol solution.

Sampling involved, light swabs of the vagina with a sterile cotton-tipped applicator, light brushing of the vaginal wall to collect more adherent interstitial microbes and a minimal lavage of vaginal canal using saline buffer. **Swab**: A sterile cotton swab was aseptically inserted 0.5–2 cm into vaginal canal and rotated 360° while sampling a large portion of the outer 1/3 of the tract. The swab tip was subsequently placed into a collection tube containing 1 ml sterile saline solution (0.9% NaCl). Vaginal scrapings were collected primarily for recovery of more adherent microbial species. A small sterile brush was inserted 0.5–1.5 cm in the vagina, used to gently scrape a large portion of the lateral wall vaginal epithelium, placed into a vial containing 1 ml sterile saline solution, and gently agitated to remove the collected material. Finally, a small diameter sterile pipette was inserted 0.5–2 cm into the vaginal canal, saline injected and suctioned back into the pipette. The recovered saline solution was transferred to a sample tube. All samples were immediately frozen after collection.

Vaginal sample DNA extraction

Total DNA was isolated from baboon vaginal samples by concentrating the cells by centrifugation, rinsing them once with 500 µl of 0.9% NaCl , followed by resuspention in 500 µl of 5X lysis solution (0.5 M Na•EDTA, pH 8, and 75 mg/ml lysozyme, added immediately before use). Three cycles of freeze-thaw were applied each consisting of 5 min freezing in a dry ice/ethanol slurry, and 5 min thawing at 37° C. After the final thaw, the sample was incubated for 30 min at 55° C. Subsequently, 70 µl of 5 M NaCl was added, and the resulting solution

incubated on ice for 30 min followed by centrifugation for 20 min at 14,000 rpm. Genomic DNA containing supernatant was transferred to clean tubes, 750 µl cold 1M TE saturated phenol pH 8, was added and mixed thoroughly for 1 min. Centrifugation at 14,000 rpm was performed for 10 min to separate phases, and the aqueous phase was transferred to a new tube. To maximize DNA yield we included a back extraction step consisting of the addition of 250 µl of 0.5 M NaOAc (diluted in TE buffer) was added to the remaining phenol phase. Following 15 sec of vortex mixing and centrifugation for 5 min at 14,000 rpm, the aqueous phase was pooled with that obtained previously. Phenol:chloroform:isoamyl alcohol (25:24:1) was added to the pooled aqueous phases, mixed for 30 sec, and centrifuged for 5 min at 14,000 rpm. The aqueous phase was transferred to a new tube containing chloroform, mixed, and centrifuged for 5 min. The aqueous phase from each tube was split into two new tubes and 2 volumes of cold 100% ethanol were added. This mix was incubated at –20° C for 24 h, after which the samples were centrifuged at 4° C (14,000 rpm) for 30 min to pellet the DNA. The pellet was washed with cold 70% ethanol and air dried for 15 min. Genomic DNA was resuspended in Tris•EDTA and stored at –20° C for later use.

PCR amplification

Amplification for DGGE

The variable V3 region of the 16S rDNA was amplified using primers to conserved regions 341 and 534 of the 16S rRNA gene. Oligonucleotide

sequences are as follow: primer 16SV3f, 5'-CCTACGGGAGGCAGCAG-3'; and primer 16SV3r, 5'-ATTACCGCGGCTGCTGG-3'. Primer 16SV3f contains an additional 40 nucleotide GC-rich region at its 5' end that serves as a GC clamp. Each amplification consisted of 2.5 µl 10x PCR+$MgCl_2$ buffer (200 mM Tris-HCl, pH 8.4, 500 mM KCl, 2 mM $MgCl_2$), 1 mg/ml bovine serum albumin (Invitrogen), 250 µM dNTPs, 1 µM of each primer (IDT, Coralville, IA), 1.25 U of FastStart *Taq* DNA polymerase (Roche, Indianapolis, IN), 1 µl DNA sample, ultra-pure H_2O to a 25 µl final volume. Amplification reactions were carried out in 0.2 ml tubes in a MJ Research PTC Peltier thermal cycler (Bio-Rad Laboratories, Hercules, CA). The reactions were first incubated for 1 min at 94°C to denature template DNA. Subsequently 30 cycles of 1 min at 94 °C, 1 min at 61 °C and 1 min at 72 °C were performed. A final extension step was carried out at 72°C for 7 min. Amplification products were diagnosed by agarose (1%) gel electrophoresis.

Denaturing gradient gel electrophoresis (DGGE)

DGGE based on the protocol described by Simpson et al. (17) was performed on a DCode apparatus (Bio-Rad Laboratories, Hercules, CA). An 8% polyacrylamide gel (acrylamide: bisacrylamide 37.5:1) in 1x Tris-acetate EDTA (1x TAE = 40 mM Tris-acetate pH 8, 1 mM EDTA) buffer with a denaturing gradient of 35-60% urea-formamide (100% urea-formamide contains 7 M urea and 40% deionized formamide) was loaded with 10 µl PCR amplified product from baboon vaginal samples. Electrophoresis was performed for 16 h at 60 °C at a constant 70 V. After electrophoresis gels were soaked in fixation buffer (1x

TAE, 10% ethanol and 0.5% acetic acid) and stained for 15 min in a TAE staining solution containing 0.1% silver nitrate. Stained gels were washed once with ultra-pure distilled water (NanoPure System, Millipore Billerica, MA) and subsequently soaked in a developing solution containing 0.01% sodium borohydride, 1.5% sodium hydroxide and 0.15% formaldehyde for 10 to 15 min. Gels were transfer to cellulose paper, air-dried and photographed using a digital camera system with imaging software (Bio Rad Hercules, CA). Analysis of DGGE fingerprints was performed with the Diversity Database software (BioRad Laboratories, Hercules, CA).

RESULTS

PCR-DGGE showed separation patterns with several distinguishable bands suggesting the presence of a complex microbiota, likely to be constituted of numerous different bacterial species. A representative DGGE profile for 7 baboon subjects is shown in Figure 2.1; the DGGE profiles of the remaining subjects are shown as supplemental material.

DGGE analysis reveals significant inter-individual variation

In the present study we have obtained DGGE profiles from three different samples from each of the baboons (*n*=35) studied. Two striking features are immediately evident. First, the profiles obtained from the three types of samples are notably similar in most of the animals (Figure 2.2i). This is not consistent with the possibility that sampling differences from one animal to another are

major contributors to intra-individual variations. Small differences between each sampling technique profiles are observed in the form of presence or absence of specific bands or weaker bands in some samples that may be present in other more definably (Figure 2.2ii). The differences are most evident in the lavage. We are compelled to infer that vaginal communities were sufficiently perturbed by the other two techniques to yield a more variable constitution in the lavage samples. It has been reported previously that the use of vaginal lavage is a less efficient technique for sampling of this type (3). The second striking feature is the amount of animal-to-animal variation when comparing two different baboon sample fingerprints. Assuming that the DGGE patterns reflect the true diversity of the microbial population, it appears that the baboons differed from each other both in complexity of the microbiota and in the types of microbes that dominated the site (Figure 2.4). Nonetheless, we observed particular bands to be common between subjects, which lead us to believe that these primates share a set of bacterial species. To further support these observations we generated dendrograms using UPGMA analyses of the generated matrices from obtained distance coefficients values so to determine if the banding patterns similarities of bacterial communities in each sample resulted in clustering patterns that could be related to baboon vaginal microbiota complexities. Comparison results are a set of binary outcomes reflecting the presence or absence of bands from each subject when compared to the complete (all samples) set of bands. Similarity clusters and community percent similarities were calculated based on Dice Coefficient similarity measures for each pairwise comparison and dendrograms generated

from the dissimilarity matrix. Clustering results further indicate remarkable similarities within sampling technique for each baboon (Figure 2.3). Observed similarities averaged 90% and in some baboon samples ranged closer to 95%. Conversely, clustering analyses suggest an even more remarkable variation from one baboon to another (Figure 2.4). Clustering analysis of all 35 subjects suggests relevant high inter-individual variation with average similarities ranging under 60%. Although no age, menstrual state or vaginal pH relationship between these two subjects was observed, two specific baboons (subjects 4 and 5) were observed to cluster together with similarities of up to 75%. Interestingly no specific clustering correlation to host physiological features like vaginal pH or menstrual state was observed among any of the subjects evaluated in this study. No baboon sample clustered close enough to be considered similar in microbial composition, DGGE fingerprints across all animals studied were considered unique to it subject suggesting microbial composition can potentially be a host specific relationship.

Evaluation of DGGE analysis as an accurate indicator of the complexity of the bacterial communities

In a separate study we have done an extensive 16S rRNA gene sequence analysis of the microbiota of 4 of the individuals included in this survey (16). This gave us an unusual opportunity to compare the two approaches, something that has not been done previously. Sequence information allowed us to obtain a more accurate molecular census of species present in these samples and comparisons in terms of diversity between baboon subjects was performed.

All primer sequences were removed from the consensus DNA sequences prior to analysis, so that all phylogenetic information came from regions internal to the primers and thus did not reflect minor degeneracy from the primers themselves. Moreover, in several cases specific bands that were weakly visible in DGGE gels corresponding to pools of samples was noticeably enriched in specific fractions (Figure 2.2ii). The basis of this enrichment is that the reduced complexity and increased relative abundance of templates in the individual samples compare to the DNA pool for each sample allowed templates that were present in low abundance or poorly amplified in the total community to be amplified more effectively.

DISCUSSION

The group of baboons included in this study is a unique resource due to the homogeneous nature of their environment and the genetically inbreeded makeup of this captive colony. If any group were to have minimal animal-to-animal variation, this would be expected to be an excellent candidate. Originally of interest due to the use of baboons as models for endometriosis and other female urogenital conditions, the availability of these samples allowed us the opportunity to assess individual-to-individual variation in members of such a homogeneous environment. A surprising result in our study was that even in such a population, there was considerable individual-to-individual variation. The use of DGGE analysis allowed us to evaluate samples from a large enough number of animals to draw conclusions about inter-individual variation.

It is significant that in most cases, the different types of specimens taken from the same individual had similar or almost identical patterns. This means that inconsistencies in sample collection were not responsible for the observed variation between individuals. Our results show convincingly that although DGGE does not provide species names, there was a clear difference in the community structures of different individuals. A hypothesis suggested by our results is that when an animal is born and begins to acquire its vaginal microbiota, a specific group of the many species to which the animal is exposed are acquired and maintained. This suggests that individual physiological peculiarities may have a greater impact on defining the microbiota than was previously suspected to occur in the vaginal tract. Observations obtained from studies performed in rats and rhesus monkeys have eluted to the effect of early life stresses and sudden environmental changes in the gut microbial composition (1, 15). Human studies also suggest gastrointestinal microbiota shifting as dominant bacterial taxa changes due to emotional stress (9).

The fact that some of these animals had their microbiota analyzed by a 16S rRNA cloning and sequencing analysis made it possible to assess how accurately DGGE analysis reflects the diversity of the community. Our results show that DGGE did provide in most cases an accurate qualitative gauge of the complexity of the microbial community of these baboons. Moreover, it also reinforces our observations with regards to dissimilarities from one subject vaginal microbial structure to the next. It is noteworthy to point out that the vaginal tract microbial structure is not as strongly influenced by diet or other

external factor as the gastrointestinal tract is suggested to be (4, 12). However, the fact that no effect was observed from dietary intake although for these baboons diet was fairly uniform, suggests other physiological aspects to be at play. Immune responses have been considered as an important part of commensal-host interactions for the establishment of taxonomic groups colonization of this niche (2). Evidence to support this phenomena is scarce, thus more studies on host physiological response in particular primates are needed to define the nature of these interactions.

REFERENCES

1. **Bailey, M. T., and C. L. Coe.** 1999. Maternal separation disrupts the integrity of the intestinal microflora in infant rhesus monkeys. Dev Psychobiol **35:**146-55.

2. **Blum, S., S. Alvarez, D. Haller, P. Perez, and E. J. Schiffrin.** 1999. Intestinal microflora and the interaction with immunocompetent cells. Antonie Van Leeuwenhoek **76:**199-205.

3. **Delany, S., R. Rosas, N. Mlaba, T. Clayton, G. Akpomiemie, J. Legoff, A. Capovilla, L. Belec, W. Stevens, and P. Mayaud.** 2008. Comparison of Cervicovaginal Lavage, Cervicovaginal Lavage Enriched With Cervical Swab, and Vaginal Tampon for the Detection of HIV-1 RNA and HSV-2 DNA in Genital Secretions. J Acquir Immune Defic Syndr.

4. **DiBaise, J. K., H. Zhang, M. D. Crowell, R. Krajmalnik-Brown, G. A. Decker, and B. E. Rittmann.** 2008. Gut microbiota and its possible relationship with obesity. Mayo Clin Proc **83:**460-9.

5. **Ferris, M. J., J. Norori, M. Zozaya-Hinchliffe, and D. H. Martin.** 2007. Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. J Clin Microbiol **45:**1016-8.

6. **Flint, H. J., S. H. Duncan, K. P. Scott, and P. Louis.** 2007. Interactions and competition within the microbial community of the human colon: links between diet and health. Environ Microbiol **9:**1101-11.

7. **Forney, L. J., J. A. Foster, and W. Ledger.** 2006. The vaginal flora of healthy women is not always dominated by *Lactobacillus* species. J Infect Dis **194:**1468-9; author reply 1469-70.

8. **Hastings, J. M., and A. T. Fazleabas.** 2006. A baboon model for endometriosis: implications for fertility. Reprod Biol Endocrinol **4 Suppl 1:**S7.

9. **Holdeman, L. V., I. J. Good, and W. E. Moore.** 1976. Human fecal flora: variation in bacterial composition within individuals and a possible effect of emotional stress. Appl Environ Microbiol **31:**359-75.

10. **Hyman, R. W., M. Fukushima, L. Diamond, J. Kumm, L. C. Giudice, and R. W. Davis.** 2005. Microbes on the human vaginal epithelium. Proc Natl Acad Sci U S A **102:**7952-7.

11. **Ley, R. E., F. Backhed, P. Turnbaugh, C. A. Lozupone, R. D. Knight, and J. I. Gordon.** 2005. Obesity alters gut microbial ecology. Proc Natl Acad Sci U S A **102:**11070-5.

12. **Ley, R. E., P. J. Turnbaugh, S. Klein, and J. I. Gordon.** 2006. Microbial ecology: human gut microbes associated with obesity. Nature **444:**1022-3.

13. **Marteau, P., P. Pochart, J. Dore, C. Bera-Maillet, A. Bernalier, and G. Corthier.** 2001. Comparative study of bacterial groups within the human cecal and fecal microbiota. Appl Environ Microbiol **67:**4939-42.

14. **Moore, W. E., and L. V. Holdeman.** 1974. Human fecal flora: the normal flora of 20 Japanese-Hawaiians. Appl Microbiol **27:**961-79.

15. **O'Mahony, S. M., J. R. Marchesi, P. Scully, C. Codling, A. M. Ceolho, E. M. Quigley, J. F. Cryan, and T. G. Dinan.** 2008. Early Life Stress Alters Behavior, Immunity, and Microbiota in Rats: Implications for Irritable Bowel Syndrome and Psychiatric Illnesses. Biol Psychiatry.

16. **Rivera, A. J., J. Frank, R. Stumpf, A. Salyers, B. Wilson, G. Olsen, and S. Leigh.** 2010. Differences Between the Vaginal Microbiota of Baboons and That of Humans. American Journal of Primatology. **71:**1-8.

17. **Simpson, J. M., V. J. McCracken, B. A. White, H. R. Gaskins, and R. I. Mackie.** 1999. Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. J Microbiol Methods **36:**167-79.

18. **Stevens, V. C.** 1997. Some reproductive studies in the baboon. Hum Reprod Update **3:**533-40.

19. **Sullivan, A., C. Edlund, and C. E. Nord.** 2001. Effect of antimicrobial agents on the ecological balance of human microflora. Lancet Infect Dis **1:**101-14.

20. **Turnbaugh, P. J., F. Backhed, L. Fulton, and J. I. Gordon.** 2008. Diet-induced obesity is linked to marked but reversible alterations in the mouse distal gut microbiome. Cell Host Microbe **3:**213-23.

21. **Walker, A. W., S. H. Duncan, E. C. McWilliam Leitch, M. W. Child, and H. J. Flint.** 2005. pH and peptide supply can radically alter bacterial populations and short-chain fatty acid ratios within microbial communities from the human colon. Appl Environ Microbiol **71:**3692-700.

22. **Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney.** 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology **150:**2565-73.

FIGURES

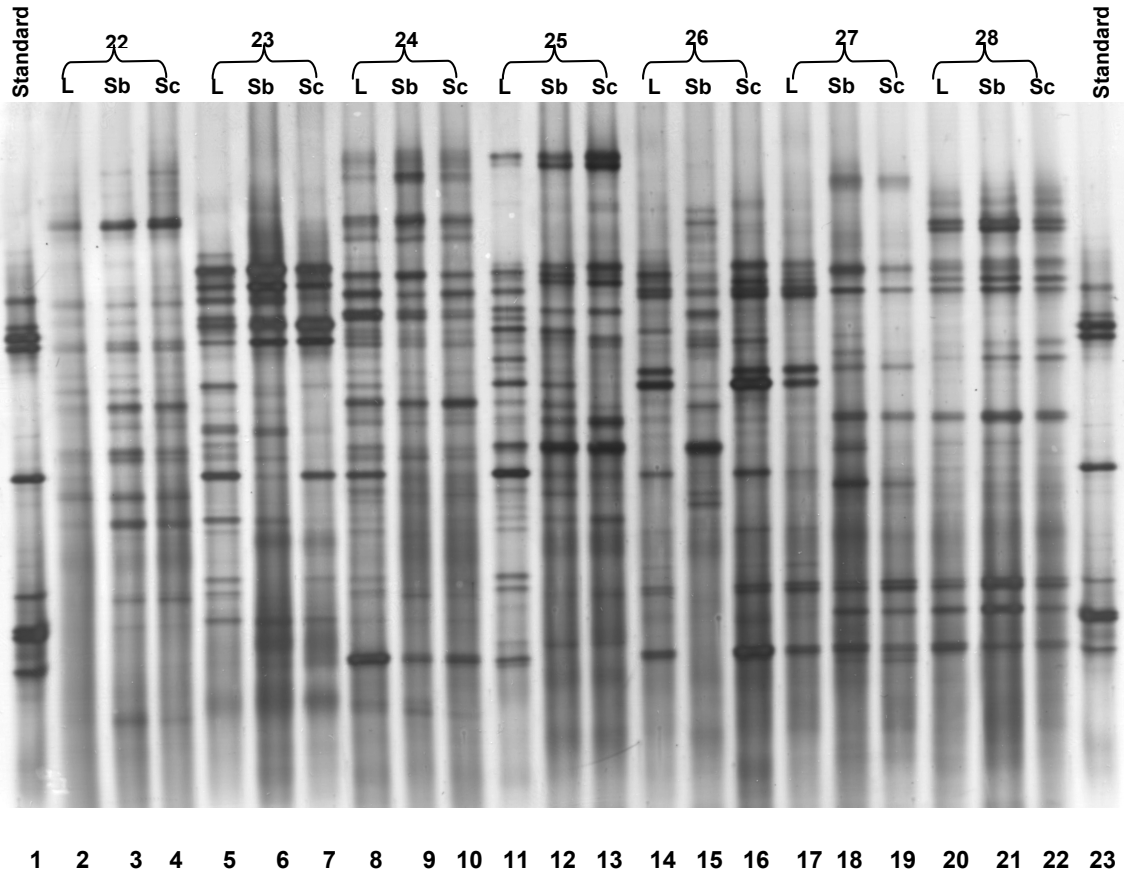


Figure 2.1 Negative image of a silver nitrate-stained perpendicular DGGE separation pattern of 21 samples from seven baboons. PCR-DGGE fingerprints of the V3 region of the 16S rDNA (lanes 2 to 22) for Lavage (L), Swab (Sb) and Scrape (Sc) sampling techniques showed remarkable similarities, (e.g.: lane set 2-4). Subjects 22-28 presented here exhibit greater inter-individual variation than intra-individual variation (even though the latter represent different sampling methods, rather than technical replicates).

Figure 2.2

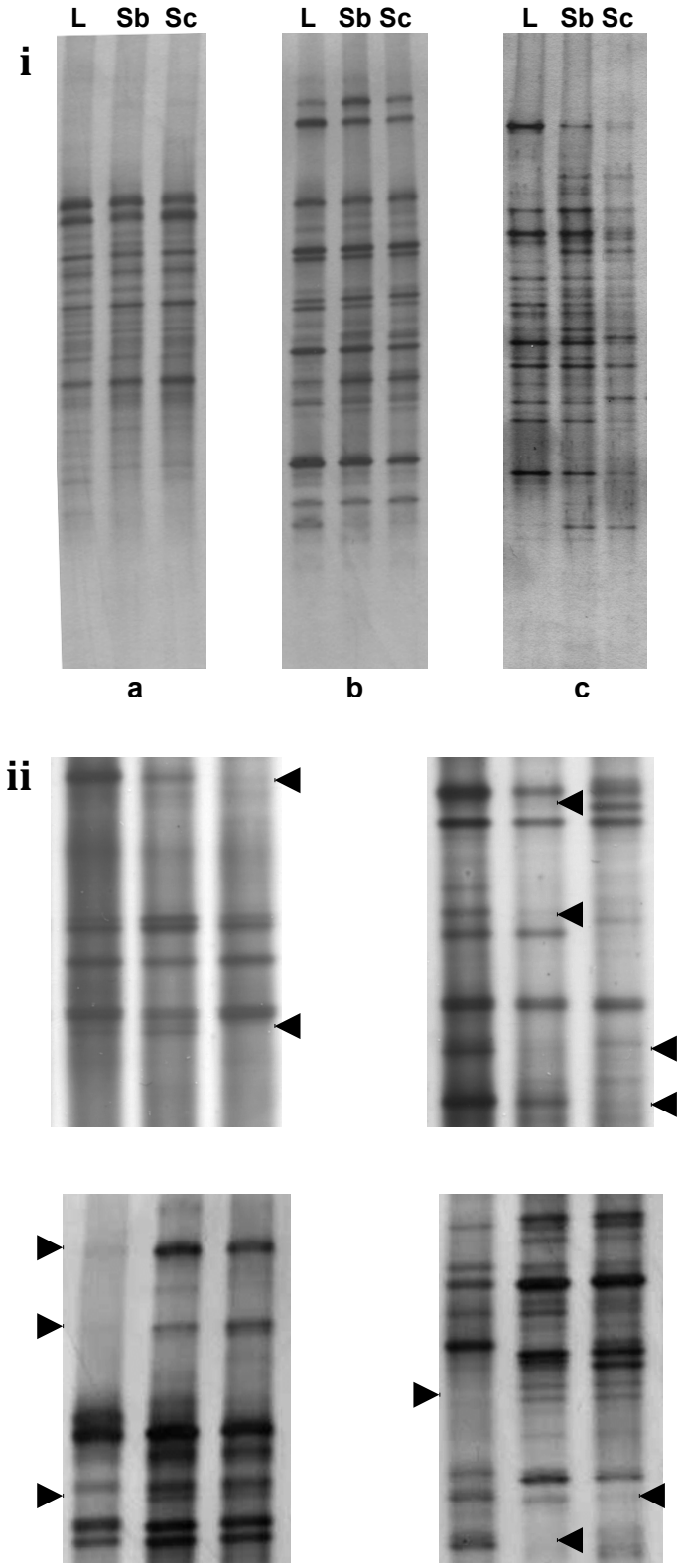


Figure 2.2 (cont.)

Figure 2.2 (**i**) Most commonly, a high degree of similarity is observed among samples from the same individual. Patterns **a**, **b** and **c** represent three different examples of the remarkable similarities observed between the different sampling techniques used in this study. Patterns appear to be almost identical with particular bands showing higher intensity than others. (**ii**) Arrows point out particular examples of discontinued banding relating to bands that were weakly visible in DGGE gels corresponding to pools of samples noticeably enriched in other specific fractions.

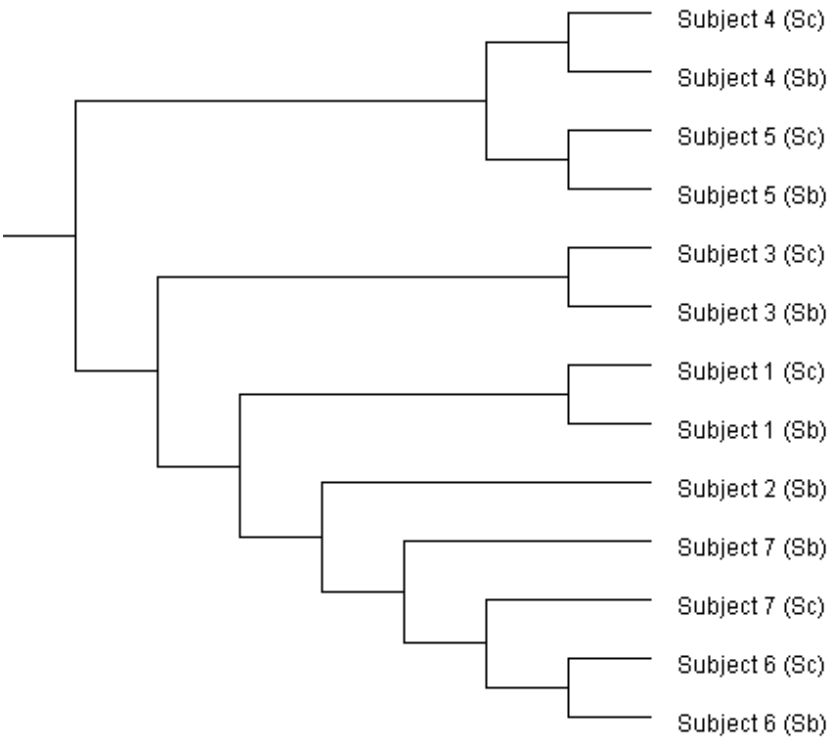


Figure 2.3 Dendrogram representing relationships between PCR-DGGE fingerprints from scrape (Sc) and swab (Sb) samples of 7 subjects. Clustering showed high similarities within sampling technique for each subject. Amplification of scrape samples from subject 2 failed to produce a fingerprint and was excluded from this comparison. Dendrogram was constructed based on migrational distances of the V3-16s rDNA PCR product within each DGGE gel.

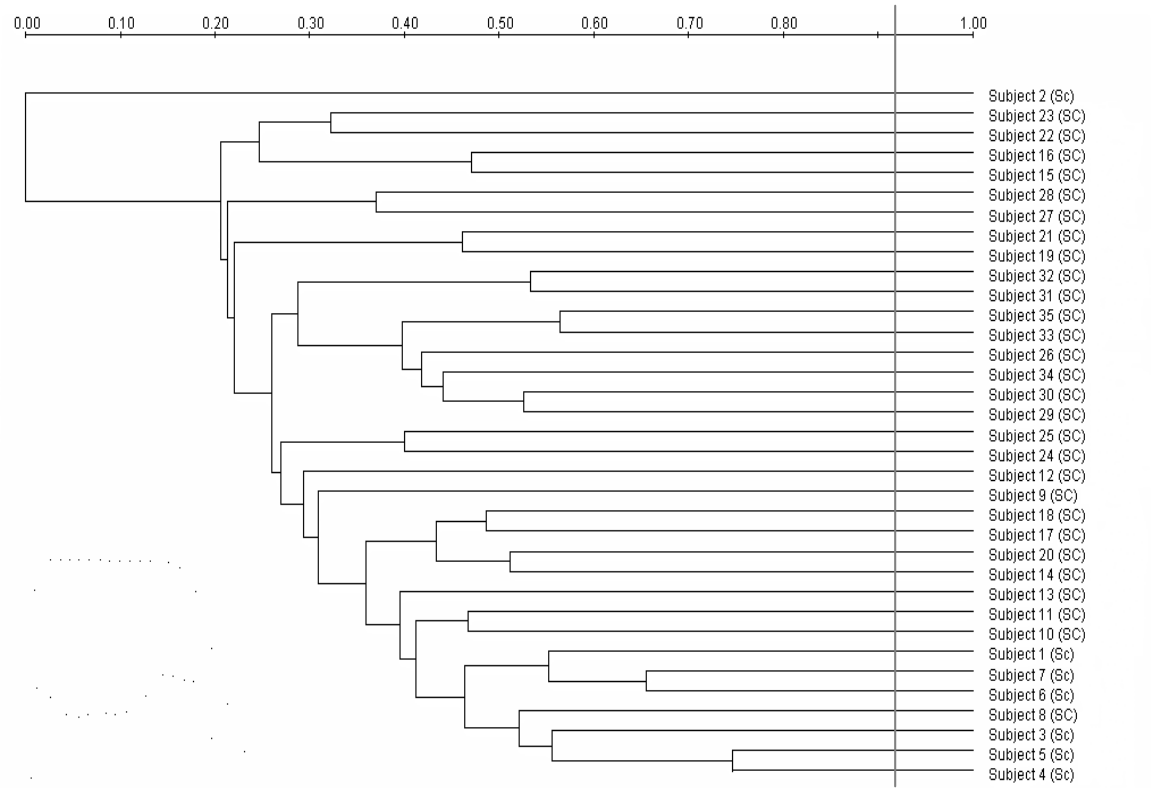


Figure 2.4 Percent similarity clustering analyses for all 35 baboons in this study. High degree of inter-individual variation is observed for all 35 baboons. As a similarity index reference point and measure of significance, we compared two identical banding patterns produced by the gel ladder generating a cutoff line at 93%. Clustering suggest significant differences (< 60%) in all subjects microbiota structure with the exception of baboons 4 and 5 (~75%) and 6 and 7 (~65%).

CHAPTER 3

MAJOR DIFFERENCES BETWEEN THE VAGINAL MICROBIOTA OF BABOONS AND HUMANS

INTRODUCTION

The relationship between mammals and their resident microbes remains poorly understood, but it has been generally assumed that closely related host species are more likely to have a similar microbiota than more distantly related host species (14). If this assumption is correct, nonhuman primates should have a microbiota whose composition is similar to that of humans. Although *Papio* baboons (hereafter termed simply "baboons") are not as closely related to humans as the great apes, the physiology of the baboon urogenital tract has been considered to be similar enough to that of humans to promote the use of baboons as non-human models for human reproductive disorders (2, 6, 17). For example, baboon menstrual cycles are similar to those of humans in duration and hormonal profiles (19). In addition, like humans, baboon neonates have large brains (9), potentially posing obstetric challenges comparable to those faced by humans.

Despite similarities in anatomy and physiology, differences have been observed. For example, the baboon vaginal tract pH is somewhat higher (pH 6-7) than the pH of the human vagina (<5) (12, 13, 20). Whether this is due to host physiology or to bacterial activities is unknown. Although there is considerable

information about the anatomy and physiology of the baboon reproductive tract, virtually nothing was known about the microbiota of the vaginal tract. Except for one 1979 cultivation-based study of the baboon vaginal microbiota by Skangalis et al, (18) no further analysis of the baboon vaginal microbiota has been available. This one study was intriguing because it suggested that taxa such as the Bacteroidetes, which are not generally found in the healthy human vagina, are found in baboons. Nonetheless, Skangalis et al. identified lactobacilli as one of the major groups present in the baboon vagina, a feature of the vaginal microbiota that made them appear similar to humans.

Here we report the first analysis of the baboon vaginal microbiota based on 16S rRNA gene sequences. The results of our analysis suggest that there are significant differences between human and baboon microbial composition previously not observed and vastly underestimated.

MATERIALS AND METHODS

Sample collection

Vaginal specimens were taken from nine adult female baboons (*Papio hamadryas*) residing in the Southwest National Primate Research Center (SNPRC), San Antonio, Texas and six wild adult female baboons from the Amboseli Baboon Research Project (ABRP) in Amboseli, Kenya.

Captive baboons

Vaginal tract sample collection was performed following approved IACUC protocols. All primates received comparable diet and they were housed in proximity to one another. The baboons studied were of a similar genetic background because they are mostly descendants of a modest number of founders (24). To avoid cross-contamination of samples with fecal or soil bacteria, the concrete floor of the cage area was regularly cleaned with hoses.

Wild baboons

Mature females in this study were members of five social groups in the ABRP baboon population and subsisted entirely on wild foods in a semi-arid habitat (mean rainfall = 346.5 mm per year) (26). They had minimal contact with human settlements and rarely obtained any scraps of food in the event of an encounter. They were never fed, and are not handled in any way, except on the occasions when they were darted for the obtention of samples including this study. Most of the subjects in this study had never been darted before. Wild baboon sampling was performed according to Princeton University (#1547) and Duke University (#A1830-06-04) IACUC protocols.

For each captive subject, three kinds of samples were taken: a light swab taken with a cotton swab, a gentle scrape taken with a sterile spatula, and a final lavage consisting on the injection of saline buffer into the vaginal canal and subsequent aspiration for collection. The three different techniques were used to collect microbes with different levels of adherence to the vaginal epithelium (8).

DGGE analysis indicated that there were virtually no differences between the bacterial communities harvested by these different collection methods. For wild baboons only swabs samples were collected. Accordingly, we focused primarily on the swab samples. Moreover, using denaturing gradient gel electrophoresis (DGGE) analysis we had ascertained that swabs and scrape samples were virtually identical (data not shown).

None of the baboons chosen received antibiotic treatment during several months prior to our sampling. Primates were sedated for sampling using an intra-muscular injection of ketamine hydrochloride at a dosage of 10-15 mg/kg bodyweight. Upon sample collection, the swab tip was placed into a collection tube containing 1ml sterile saline solution and the spatula submerged and agitated to dislodge any collected material. All samples were immediately frozen after collection. Swab samples obtained from wild baboons were placed in 1 ml RNA/*ater* solution.

Vaginal sample DNA extraction

Total DNA was isolated from baboon vaginal samples by concentrating cells and rinsing with 500 µl of 0.9% NaCl solution followed by the immediate addition with 5X lysis solution [0.5 M Na•EDTA, pH 8, and 75 mg/ml lysozyme (Sigma-Aldrich, St. Louis, Missouri)]. Three cycles of freeze-thaw were applied each consisting of 5 minutes freezing in ice/ethanol 200 proof slurry, 5 minutes thawing at 37° C and a final incubation period of 30 minutes at 55° C. Immediately after lysis, 5M NaCl was added and incubated on ice for 30 minutes

followed by centrifugation at 14,000 rpm. Genomic DNA containing supernatant was transferred to clean tubes, cold Tris•EDTA saturated Phenol (Fisher Scientific) was added and mix thoroughly for 1 minute. Centrifugation at 14,000 rpm was performed for 10 minutes to separate phases from which the soluble phase is transferred to a new tube. To maximize DNA yield we implemented a back extraction step consisting of the addition of 0.5 M NaOAc to the TE in the remaining phenol phase. The mix was vortexed for 15 seconds, centrifuged for 5 minutes at 14,000 rpm and the aqueous phase added to initial phase obtained prior to back extraction. Phenol:chloroform:isoamyl alcohol (25:24:1) was added to the pooled aqueous phases mix for 30 seconds and centrifuged for 5 minutes at 14,000 rpm. The aqueous phase was transferred to new tubes containing chloroform and centrifuged for 5 minutes. Aqueous phase in each tube is separated into two new tubes and 2 volumes of cold 100% ethanol are added. This mix is incubated at – 20° C for a minimum of 2 hours. To maximize yield, DNA was incubated for 24 hours after which DNA is centrifuged at 4° C (14,000 rpm) for 30 minutes to pellet DNA. Nucleic acid pellet was washed with cold 70% ethanol and air dry for no less than 15 minutes. Genomic DNA was resuspended in Tris•EDTA and stored at – 20° C for later use.

16S rDNA amplification

Nearly complete bacterial 16S rRNA genes were amplified using the bacterial specific primer formulation 27fYM+3 and 1492r (4, 21). Reactions consisted of 25 µl PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTP mix, 200 nM each of

forward and reverse primer, and 0.25 U Platinum Taq DNA polymerase (Invitrogen). Reactions were incubated at 94°C for 4 minutes, followed by 24 cycles of denaturation at 94°C for 1 min, annealing at 48°C for 30 seconds, and elongation at 72°C for 2 min. The reactions were then held at 4°C to add reconditioning mix containing, 25 µl PCR buffer, 2 mM $MgCl_2$, 0.2 mM dNTPs, 900 nM of each forward and reverse primer and 0.25 Units of Taq polymerase. The reactions were then subjected to a final cycle consisting of denaturation at 94°C for 1 min, annealing at 48°C for 30 seconds and elongation at 72°C for 12 minutes. Reconditioning steps were incorporated to prevent or minimize heteroduplex formation and creation of artificial 16S rDNA sequences as described previously (4, 21). PCR products were purified using spin columns (Qiagen), eluted with 50 µl 1:5 diluted elution buffer (2.5 mM Tris-Cl pH 8.5) and concentrated by lyophilization. To ensure elimination of prematurely terminated and/or spurious PCR product, the concentrated DNA was resuspended in 10 µl H_2O pH 8.0 and size selected for amplicons corresponding to 1500bp by using a low melting 1% high purity Seaplaque agarose gel. The extracted bands were then gel purified (Qiagen) and resuspended in 10 µl ultra-pure H_2O.

Cloning and sequencing of amplified 16S rDNA

Cloning of PCR amplified DNA was performed using a TOPO TA cloning kit according to manufacturer instructions with exception of a minor modification consisting of the reduction of the reactions to half volumes and incubation periods performed at a constant 25°C for 20 min. Transformants were plated into

Luria-Bertani agar plates containing 50 µg/ml kanamycin, and incubated at 37°C for 18-24 hrs. Randomly picked isolated colonies were transferred to 96 well round bottom microtiter plates containing 150 µl Luria-Bertani broth supplemented with 50 µg/ml kanamycin and incubated at 37°C overnight. Correct insert size of approximately 1,500 bp were confirmed by PCR amplification using M13 forward and reverse primers that anneal to the cloning vector flanking our inserts.

Analysis of sequence information

Near full-length SSURNA sequence analysis was performed using a set of tools available in the ClustalW multiple sequence alignment programs (1). Global alignments of these data sets were performed using default settings to calculate distances between all pair of sequences and the Neighbor-joining method (15), was applied for the generation of a phylogenetic tree depicting 16SRNA genes relationship. Baboon sequences were compared to 438 human derived bacterial sequences (GeneBank accession #: AY958774–AY959212) from previous vaginal microbiota report (7). On thousand and two sequences from cultivated bacteria were obtained from the databases and used as a reference library.

RESULTS

Microbiota composition

Vaginal pH in baboons is higher than that normally encountered in humans, with the pH of the baboon vagina ranging from pH 6 to 8. In humans,

pH values this elevated would be interpreted to indicate a disease state called bacterial vaginosis (3, 16, 22, 25). These different pH values could reflect differences in the composition of vaginal secretions or in the activities of vaginal bacteria or both.

A comparison of the taxa we found in vaginal samples from baboons and ones reported from previous studies of samples from humans is summarized in the pie charts shown in Figure 3.1. A problem we encountered with the samples obtained from wild baboons was that samples from 3 of the 6 subjects contained *Shigella spp.* almost exclusively. Samples shipped over such large distances and through a number of transit points are prone to contamination. However, *Shigella* 16S rRNA sequences were not all the same sequences, possibly indicating that the preponderance of *Shigella* was not contamination, but since we could not rule out contamination as an explanation, we used only the 3 animals that had a more diverse microbiota in the wild baboon pie chart shown in Figure 3.1. If *Shigella spp.* proves to be predominant in the vaginal tracts of some animals that are healthy enough to survive under wild conditions, this finding would raise interesting questions about the ability of the primate body to tolerate major disruptions in the normal microbiota.

As seen in Figure 3.1, we found a preponderance of sequences belonging to the Firmicute phylum in all three groups: captive baboons, wild baboons and humans. Although the Firmicutes were composed primarily of bacilli in all three cases, most of the human Firmicutes were members of the genus *Lactobacillus* in humans, whereas the baboons harbored a diverse group of *Clostridium spp*.

All captive, wild baboon and human groups of subjects harbored Proteobacteria. These included not only pseudomonads, such as those pseudomonads in the human subjects, but also two different dominant genera of Fusobacteria (*Fusobacterium* and *Sneathia*). In humans, the Actinobacteria were primarily *Gardnerella* species. In the baboons, the Actinobacteria were, with the exception of a small subset of sequences, unidentified close and distant relatives of the *Gardnerella* species found in humans.

Phylogenetic comparison of human and baboon microbiota

More detailed results of our phylogenetic analysis of the baboon vaginal microbiota and comparison with human sequences obtained from the literature are summarized in Figure 3.2 in the form of a radial tree. The differences between the baboon and human vaginal microbiota are particularly evident in this figure.

We observed some overlap within broad phylogenetic groups of ribotypes from humans and baboons, even within these same groups, the baboon sequences generally clustered separately from those of humans. That is, sequences that database searches identified as belonging to a particular group of bacteria such as the clostridia or the Bacteroidetes generally clustered separately from the reference sequences that were obtained from human isolates. This is illustrated in Figure 3.3a, which compares the genetic distances of sequences from the human and baboon vaginal tracts from type strain reference sequences. Most of the type strains were human isolates. Within

phylogenetic groups binned at the genus level (sharing at least 93% 16S rRNA gene sequence identity), we found substantial divergence between the sequences obtained from baboons relative to those from humans and the closest-named species. Microbial 16S rDNA sequences in the human vagina diverged less than 1% from the nearest cultured relative. This was true for 62% of the human vaginal clones. By contrast, only 4% of the baboon vaginal clones were 99% identical to one of the type strains included as references and only 11% of the baboon sequences were more than 97% identical to any given type strain, indicating that most of the baboon microbes were in different species, and in some cases different genera, from those found in humans.

We had generated over twice as many baboon sequences compared to human derived bacterial sequences we had available. To determine whether this disparity might affect the analysis, we picked at random the same number of sequences for baboons as we had for humans (Figure 3.3b). After controlling for the different numbers of baboon and human sequences available, 63% of the baboon sequences were 97–100% identical to a sequence from another baboon, whereas only 4% of the baboon sequences were this similar to a human-associated bacterial sequence. In general, the human bacterial species were much more closely related to described species than the baboon species. Typically, the species within a bacterial genus have 97% or greater 16S rRNA identity, a threshold at which 83% of the baboon sequences matched a sequence in another baboon, but only 31% of them matched a sequence from a human.

These results support the hypothesis that most baboon vaginal bacteria are from genera distinct from those associated with humans.

Principal component analysis (PCA) and P Test Significance analysis based on our phylogenetic information was performed to estimate similarities between baboons and human vaginal microbial communities (11). Our PCA results showed a marked separation of the baboon-derived sequences from those of sequences humans and our P test analysis ($P < 0.01$) confirmed that this difference was significant (Figure 3.4). Among the baboons tested, only one captive and one wild baboon microbial community were observed as outliers in this analysis. These baboons were however, still significantly different from bacterial communities found in humans. Figures 3.5-3.10 summarize the data for the main phylogenetic groups found in the baboon vaginal microbiota. For simplicity, only data from 4 subjects are shown.

Firmicutes

Three main classes were found within this phylum, Clostridia, Bacilli and Mollicutes (Figure 3.5a-c), with a particularly large diversity found within the Clostridia group (Figure 3.5a). Clostridial sequences from baboon samples clustered in individual clades that diverged more than 5% from the human sequences. One exception was two clades, including close relatives to *Dialister* and *Megasphaera*, where human sequences clustered with baboon sequences. Consistent with previous reports, taxa within the Bacilli derived from human samples were dominated primarily by *Lactobacillus* species (7, 25).

Representatives of the Mollicutes were found only in the baboon-derived sequences. *Mycoplasma* spp. was the closest cultivated relatives of approximately 94% of the sequences in this group.

Bacteroidetes

Close and distant relatives of the genera *Prevotella* and *Porphyromonas* were found to be dominant in this phylum of baboon-derived sequences (Figure 3.6). Only 16% of the sequences found in the baboon Bacteroidetes were similar to those of human bacteria. Baboon-derived sequences clustered in three large clades, consisting mainly of distant relatives of *Prevotella* and *Porphyromonas* species. Sequences derived from baboon and human microbes clustered in separate clades supporting our contention that there were major differences between human and baboon vaginal community composition.

Fusobacteria

Many baboon-derived sequences were most closely related to species belonging to the genera *Fusobacterium* and *Leptotrichia* (Figure 3.7). Baboon-derived sequences were most noticeably diverse within the *Fusobacterium*-related clades. Ribotypes found in baboon samples were dominant in this phylum, with the exception of one human clone that was found to be closely related to *Sneathia sanguinegens*. The *Fusobacterium* group is an example of sequences from one animal dominating the sequence representation, although here too the other animals had representatives of this group.

Actinobacteria

Baboon-derived sequences comprised 58% of sequences within the phylum Actinobacteria, while human sequences represented 42% (Figure 3.8). Of the baboon sequences found in this group, 55% of the sequences clustered in a distinct clade that was most closely related to *Gardnerella vaginalis*. Human-derived sequences contained close relatives of *G. vaginalis* but significant divergence was observed between baboon and human sequences in this clade. A second cluster of human-derived sequences from previous studies was closely related to *Bifidobacterium* species (7). The remaining sequences for both baboon and human clustered in a distinct group. Some interdigitation was observed here, but no baboon sequences were closely related to human-derived sequences.

Spirochaetes

A number of baboon-derived sequences clustered within the phylum Spirochaetes (Figure 3.9). Most of these were related to *Treponema* species. This is apparent in a clade that constituted 9% of the sequences associated with this phylum, which were closely related to *Treponema amylovorum*. No human-derived sequences were found to have any relationship to this group. Sequences outside this mentioned clade (91%) exhibited divergences greater than 10%, suggesting the presence of uncharacterized *Treponema* related species in the baboon microbial population analyzed in this study.

Proteobacteria

Sequences associated with proteobacterial classes alpha, delta, gamma and epsilon were found (Figure 3.10). Alphaproteobacteria consisted of only two human derived sequences closely related to species *Mycoplana* and *Phylobacterium*. A baboon-derived singleton of the delta proteobacteria was found to be a close relative of the genus *Desulfovibrio*. Gamma proteobacteria consisted mainly (98%) of human derived bacterial sequences with clusters of closely related pseudomonads and a region of greater diversity where 2% was represented by baboon derived sequences. These baboon sequences are close relatives of *Shigella* species. Interestingly a closely related cluster (1-2% divergence) of sequences which closer relationship to *Bacteroides ureolitycus* were the sole constituent of the epsilon proteobacteria group. *B. ureolitycus* is genotypically related to the *Campylobacter* genus but cannot be classified as such based on proteolytic metabolism and fatty acid components (23).

DISCUSSION

Our results show that despite physiological similarities between the vaginal tracts of humans and animals, there are significant differences between the microbiota of baboons and humans. In fact, most of the normal microbiota found in the baboon vagina would be considered to indicate a disease state if found in humans. Specifically, organisms such as the large number of Gram-negative bacteria and in particular the Spirochaetes are not considered normal in humans. The fact that these microbes seem to be found widely in baboons that

appear to be healthy indicates that the primate body tolerates a distinct microbiota.

Our results show that the microbiota in the primate vaginal tract appears to be host-specific at two levels. First, many of the dominant groups of bacteria in the baboon microbiota are different from those found in humans. Second, even for sequences within the same phylogenetic group, the baboon strains clustered separately from the human strains, and were different enough to be classified as members of different genera. These two findings have important implications for understanding the dynamics of host microbe relations in the primate vaginal tract. Particularly, the lack of overlap between microbiota of baboons and humans implies that the major differences in microbiota between them are not due to co-evolution. These two primate species shared a common ancestor in the Miocene, at minimum, about 22 to 25 million years old (5). Bacterial lineages on the other hand, diverged hundreds of million years or more in the past. This is true even for strains of the same species. These results indicate that the host's vaginal tract "selects" from bacterial diversity normally found in the environment for a particular subset of microbes. This selection appears to occur at the host species level, given that we document similarities within baboons in this sample.

Perhaps the most important conclusion from our results is that the bacteria colonizing the baboon vagina are responding to differences in the physiology and biochemistry of their baboon host and probably have important evolutionary consequences. Differences in the microbiota of different primate species, if

better understood, could lead to new discoveries about the physiology and evolution of primates.

REFERENCES

1. **Chenna, R., H. Sugawara, T. Koike, R. Lopez, T. J. Gibson, D. G. Higgins, and J. D. Thompson.** 2003. Multiple sequence alignment with the Clustal series of programs. Nucleic Acids Res **31:**3497-500.

2. **Fazleabas, A. T.** 2006. A baboon model for inducing endometriosis. Methods Mol Med **121:**95-9.

3. **Ferris, M. J., J. Norori, M. Zozaya-Hinchliffe, and D. H. Martin.** 2007. Cultivation-independent analysis of changes in bacterial vaginosis flora following metronidazole treatment. J Clin Microbiol **45:**1016-8.

4. **Frank, J. A., C. I. Reich, S. Sharma, J. S. Weisbaum, B. A. Wilson, and G. J. Olsen.** 2008. Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. Appl Environ Microbiol **74:**2461-70.

5. **Glazko, G. V., and M. Nei.** 2003. Estimation of divergence times for major lineages of primate species. Mol Biol Evol **20:**424-34.

6. **Hastings, J. M., and A. T. Fazleabas.** 2006. A baboon model for endometriosis: implications for fertility. Reprod Biol Endocrinol **4 Suppl 1:**S7.

7. **Hyman, R. W., M. Fukushima, L. Diamond, J. Kumm, L. C. Giudice, and R. W. Davis.** 2005. Microbes on the human vaginal epithelium. Proc Natl Acad Sci U S A **102:**7952-7.

8. **Kim, T. K., S. M. Thomas, M. Ho, S. Sharma, C. I. Reich, J. A. Frank, K. M. Yeater, D. Biggs, N. Nakamura, R. Stumpf, S. R. Leigh, R. I. Tapping, S. R. Blanke, J. M. Slauch, H. R. Gaskins, J. S. Weisbaum, G. J. Olsen, L. L. Hoyer, and B. A. Wilson.** 2009. Heterogeneity of vaginal microbial communities within individuals. J Clin Microbiol.

9. **Leigh, S. R.** 2004. Brain growth, life history, and cognition in primate and human evolution. Am J Primatol **62:**139-64.

10. **Lozupone, C., M. Hamady, and R. Knight.** 2006. UniFrac--an online tool for comparing microbial community diversity in a phylogenetic context. BMC Bioinformatics **7:**371.

11. **Lozupone, C., and R. Knight.** 2005. UniFrac: a new phylogenetic method for comparing microbial communities. Appl Environ Microbiol **71:**8228-35.

12. **Miller, C. J.** 1994. Animal models of viral sexually transmitted diseases. Am J Reprod Immunol **31:**52-63.

13. **Miller, C. J., M. Marthas, J. Torten, N. J. Alexander, J. P. Moore, G. F. Doncel, and A. G. Hendrickx.** 1994. Intravaginal inoculation of rhesus macaques with cell-free simian immunodeficiency virus results in persistent or transient viremia. J Virol **68:**6391-400.

14. **Noguchi, K., K. Tsukumi, and T. Urano.** 2003. Qualitative and quantitative differences in normal vaginal flora of conventionally reared mice, rats, hamsters, rabbits, and dogs. Comp Med **53:**404-12.

15. **Saitou, N., and M. Nei.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:**406-25.

16. **Schwebke, J. R.** 2001. Role of Vaginal Flora As a Barrier to HIV Acquisition. Curr Infect Dis Rep **3:**152-155.

17. **Skangalis, M., C. J. Mahoney, and W. M. O'Leary.** 1982. Microbial presence in the uterine cavity as affected by varieties of intrauterine contraceptive devices. Fertil Steril **37:**263-9.

18. **Skangalis, M., C. E. Swenson, C. J. Mahoney, and W. M. O'Leary.** 1979. The normal microbial flora of the baboon vagina. J Med Primatol **8:**289-97.

19. **Stevens, V. C.** 1997. Some reproductive studies in the baboon. Hum Reprod Update **3:**533-40.

20. **Tevi-Benissan, C., L. Belec, M. Levy, V. Schneider-Fauveau, A. Si Mohamed, M. C. Hallouin, M. Matta, and G. Gresenguet.** 1997. In vivo semen-associated pH neutralization of cervicovaginal secretions. Clin Diagn Lab Immunol **4:**367-74.

21. **Thompson, J. R., L. A. Marcelino, and M. F. Polz.** 2002. Heteroduplexes in mixed-template amplifications: formation, consequence and elimination by 'reconditioning PCR'. Nucleic Acids Res **30:**2083-8.

22. **van De Wijgert, J. H., P. R. Mason, L. Gwanzura, M. T. Mbizvo, Z. M. Chirenje, V. Iliff, S. Shiboski, and N. S. Padian.** 2000. Intravaginal practices, vaginal flora disturbances, and acquisition of sexually transmitted diseases in Zimbabwean women. J Infect Dis **181:**587-94.

23. **Vandamme, P., M. I. Daneshvar, F. E. Dewhirst, B. J. Paster, K. Kersters, H. Goossens, and C. W. Moss.** 1995. Chemotaxonomic analyses of *Bacteroides gracilis* and *Bacteroides ureolyticus* and reclassification of *B*. *gracilis* as *Campylobacter gracilis* comb. nov. Int J Syst Bacteriol **45:**145-52.

24. **Vice, T. E., A. R. Rodriguez**. 1965. Care and maintenance of baboon colonies in San Antonio, Texas, and Darajani, Kenja, East Africa., p. 125-140. *In* H. Vagtborg (ed.), The Baboon in Biomedical Research., vol. 2. University of Texas Press, Austin, Texas.

25. **Zhou, X., S. J. Bent, M. G. Schneider, C. C. Davis, M. R. Islam, and L. J. Forney.** 2004. Characterization of vaginal microbial communities in adult healthy women using cultivation-independent methods. Microbiology **150:**2565-73.

26. **Zinner, D., S. C. Alberts, C. L. Nunn, and J. Altmann.** 2002. Evolutionary biology: significance of primate sexual swellings. Nature **420:**142-3; discussion 143.

FIGURES
Figure 3.1

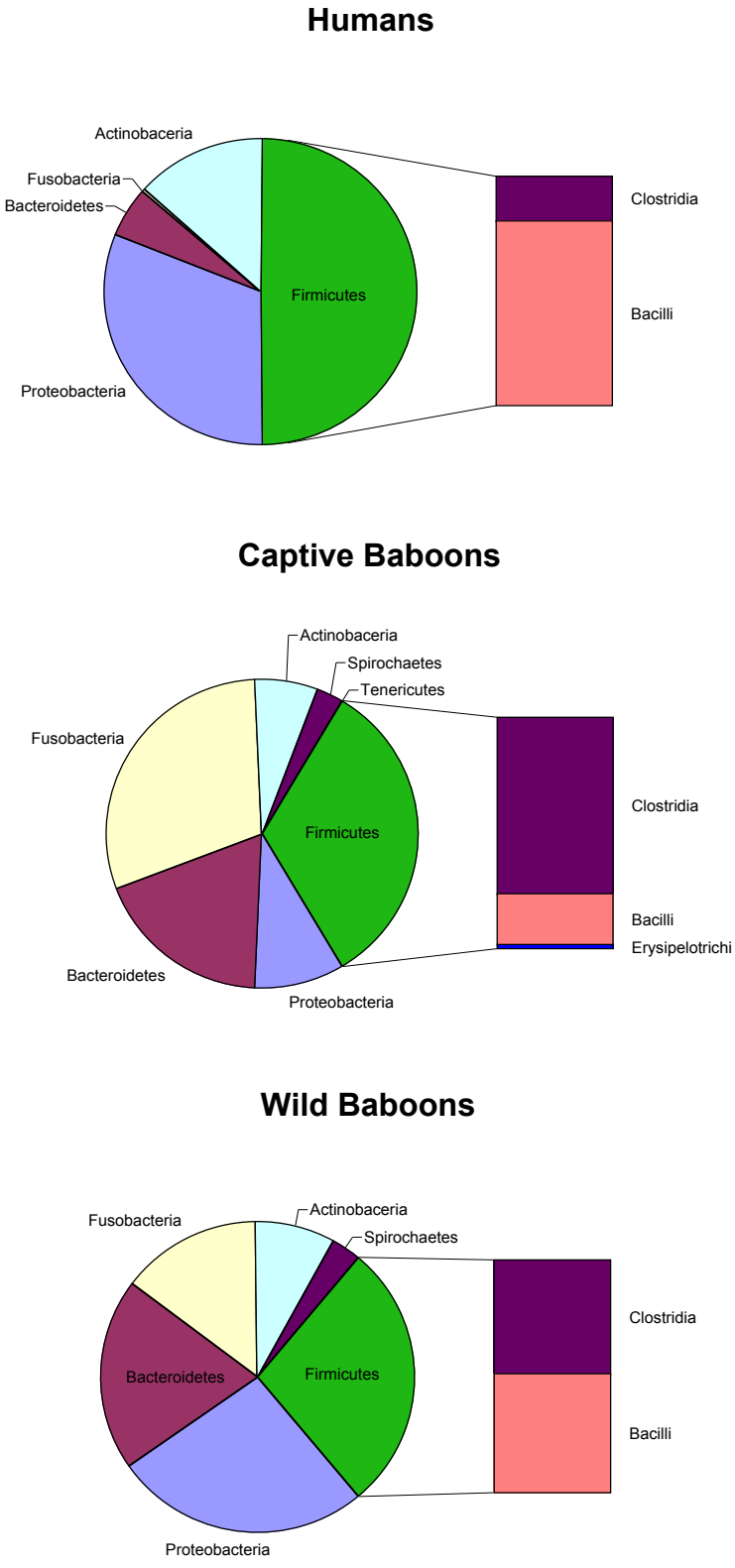


Figure 3.1 (cont.)

Figure 3.1 Phyla distributions of the vaginal tract samples across hosts. The distribution of phyla in each 16S rDNA library (from human, captive baboon and wild baboon vaginal samples) where the subdivision to the side represents taxa distributions within the Firmicutes group for each sample set. Distributions were determined by the application of the Ribosomal database project (RDP) classifier tool. Confidence thresholds were set at 95%.

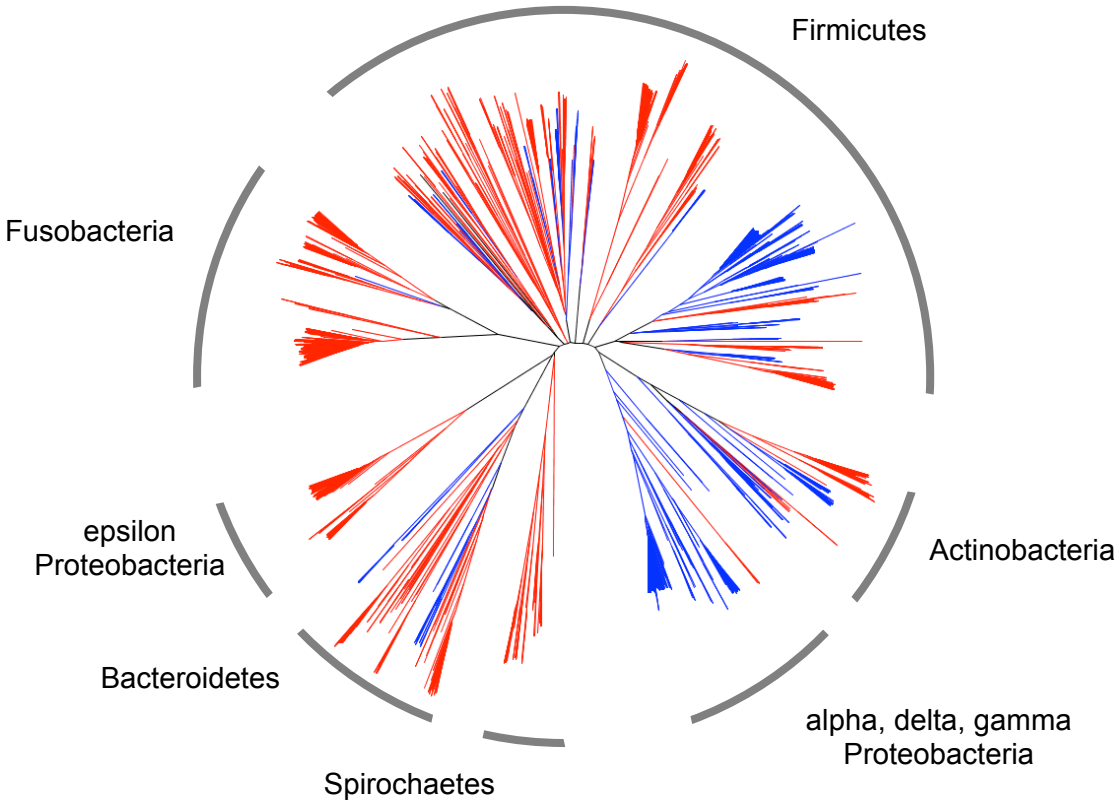


Figure 3.2 Rooted phylogenetic tree calculated by neighbor joining clustering algorithm showing the affiliation of vaginal samples species to six major taxonomic groups. This radial tree shows relationships based on near complete16S rRNA gene sequences of clones from the baboon vaginal microbial community surveyed in this study (red) and published human vaginal sequences (blue). Firmicutes fell into three different classes: Mollicutes, dominated largely by baboon species, *Bacillus* species dominated by human bacterial species and clostridial species, which were found in both humans and

Figure 3.2 (cont.)

baboons. Proteobacteria from humans were mostly members of the alpha and gamma subgroups, whereas baboon members of this phylum were members of the delta and epsilon subgroups. Spirochaetes with sequences suggesting that they were members of the genus *Treponema* were found only in the baboon samples. Members of the Bacteroidetes found in the baboon samples were distant relatives of human *Prevotella* species, species normally found in the human mouth. Fusobacteria were found primarily in the baboon samples, with the sole exception of one human-derived clone related to *Sneathia sanguinigens*. More detailed phylogenies for each phylum found in all samples are described in Figures 3.5 - 3.10. Human sequences were obtained from published data (7).

Figure 3.3

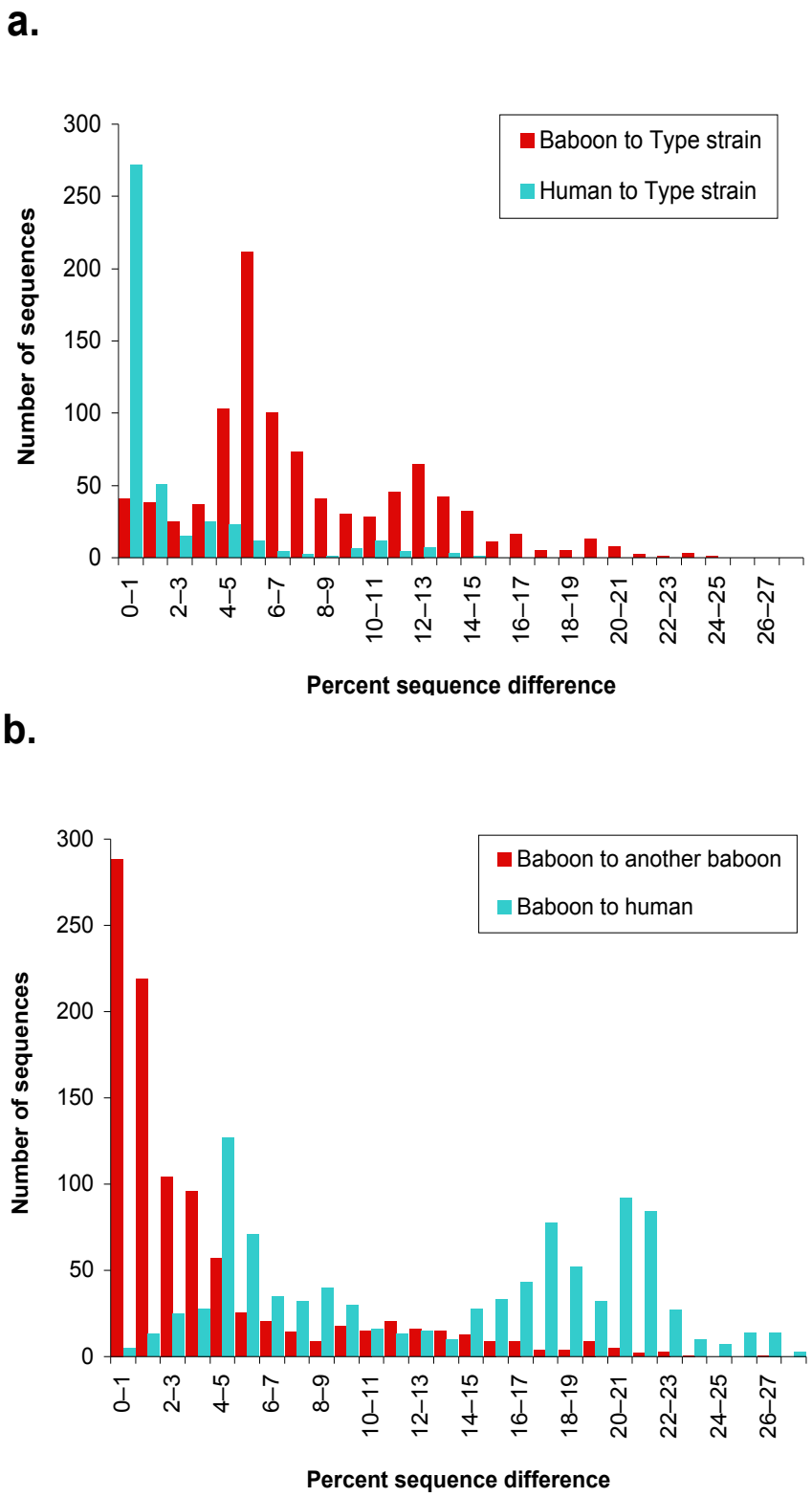

a.
Baboon to Type strain
Human to Type strain
Number of sequences
300
250
200
150
100
50
0
0–1
2–3
4–5
6–7
8–9
10–11
12–13
14–15
16–17
18–19
20–21
22–23
24–25
26–27
Percent sequence difference
b.
Baboon to another baboon
Baboon to human
Number of sequences
300
250
200
150
100
50
0
0–1
2–3
4–5
6–7
8–9
10–11
12–13
14–15
16–17
18–19
20–21
22–23
24–25
26–27
Percent sequence difference

Figure 3.3 (cont.)

Figure 3.3 6S rRNA sequence similarities of baboon and human vaginal bacteria. Panel a. Each of the baboon (red) and human (blue) vaginal bacterial sequences was scored for its highest percent similarity to a 16S rRNA sequence from a cultivated bacterial species (the Type Strain sequence, when available) (RDP; Jim Cole and Jim Garrity, personal communication; unpublished results) **Panel b.** Each rRNA sequence from the baboon samples was evaluated in terms of the most similar sequence from a baboon (red) or from a human (blue). These relationships are shown as percent identity between the rRNA gene sequences. Because there were more baboon sequences (nearly 1000) than human sequences (438), the baboon-to-baboon data are an average of results from 10 random samples (438 sequences).

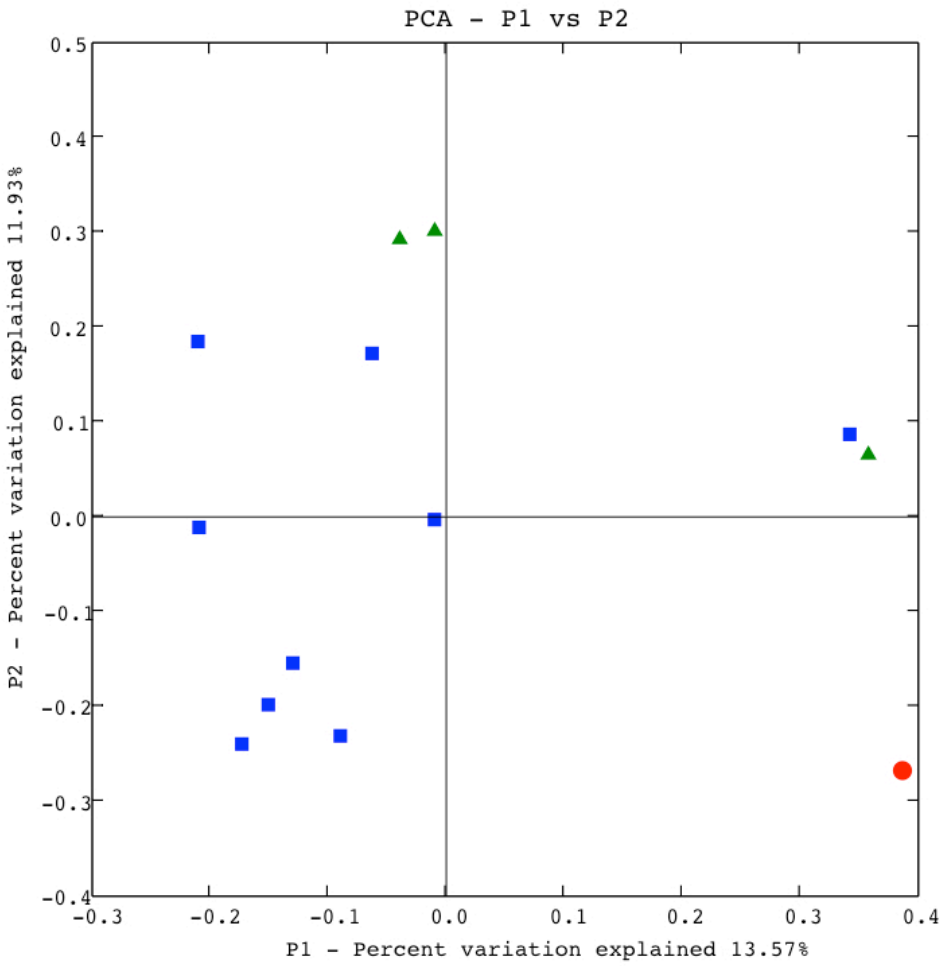


Figure 3.4 Principal component analysis (PCA) of human and baboons vaginal tract bacterial phylogenetic profiles. We used a web-based (UniFrac) interface tool to compare microbial communities extant in human and baboon vaginal tracts (10). Principal component analyses of these data ascertain community composition differences between human derived sequences (red circles) and those derived from captive (blue squares) and wild baboons (green triangles). Results suggest a high probability that each environment (host group) has more unique branch length than expected by chance. P Tests Significance

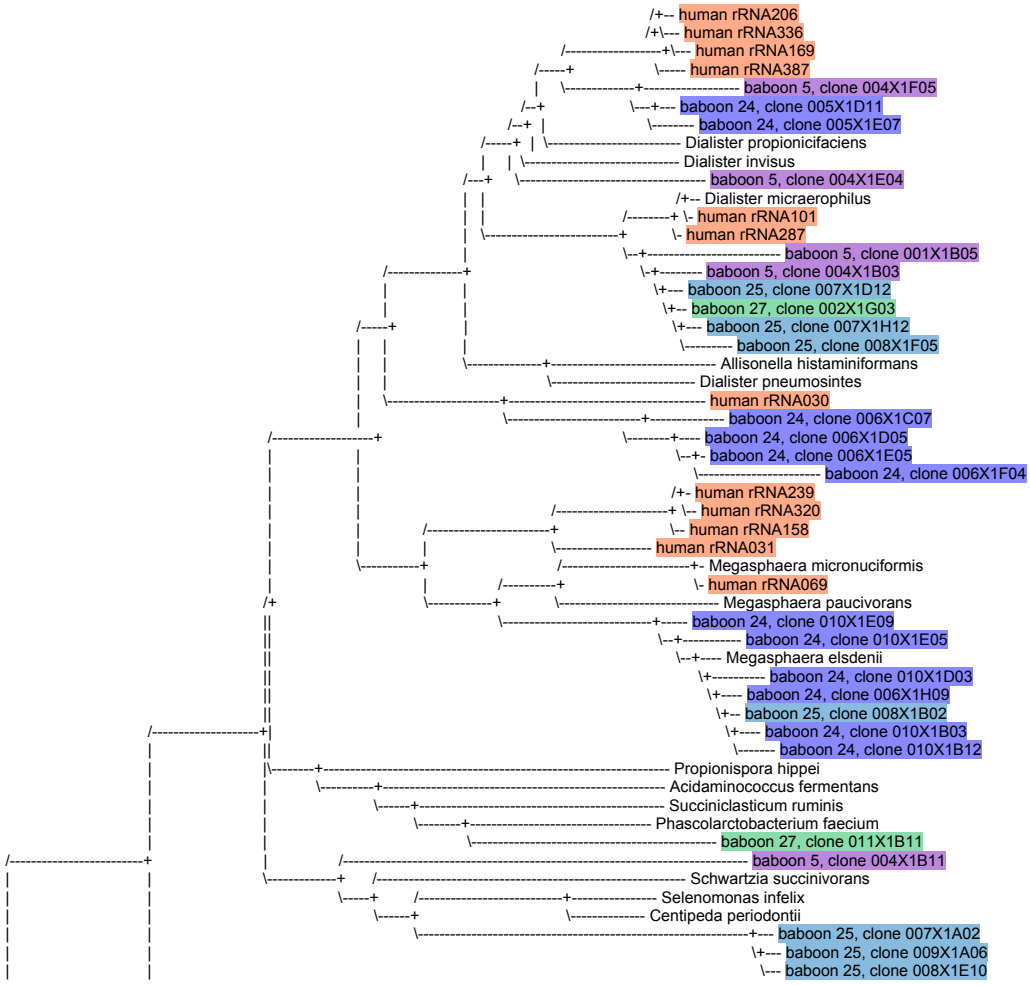
Figure 3.4 (cont.)

was also performed using 100 tree permutations from which analyses resulted in a distance *P* value of < 0.01, suggesting host bacterial composition difference to be significant. Human sequence data is represented as a single point since these data were published as a conglomerate and not assigned to its individual sample origin.

Figure 3.5

In the following figures baboon subjects are color-coded (subject 5, lavender; subject 24, dark blue; subject 25, light blue, subject 27, green). Human sequences, provided for comparison are color-coded orange. Named species from the databases (not color coded) are included as points of reference. In general, representatives of the major groups were found in all 4 of the baboon subjects, but there were cases in which a particular group was more abundant in one animal than in the others.

(A) Clostridia



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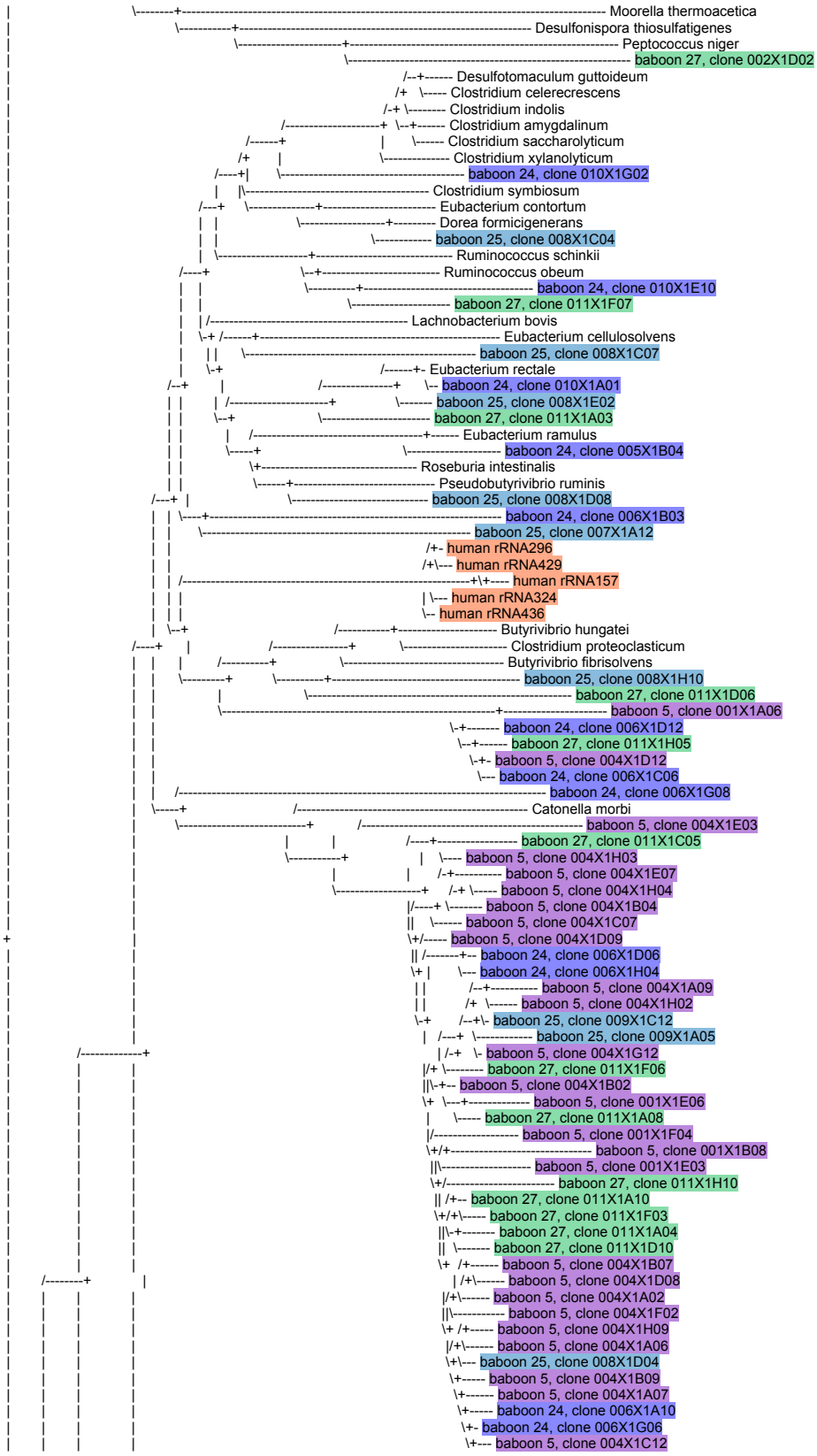

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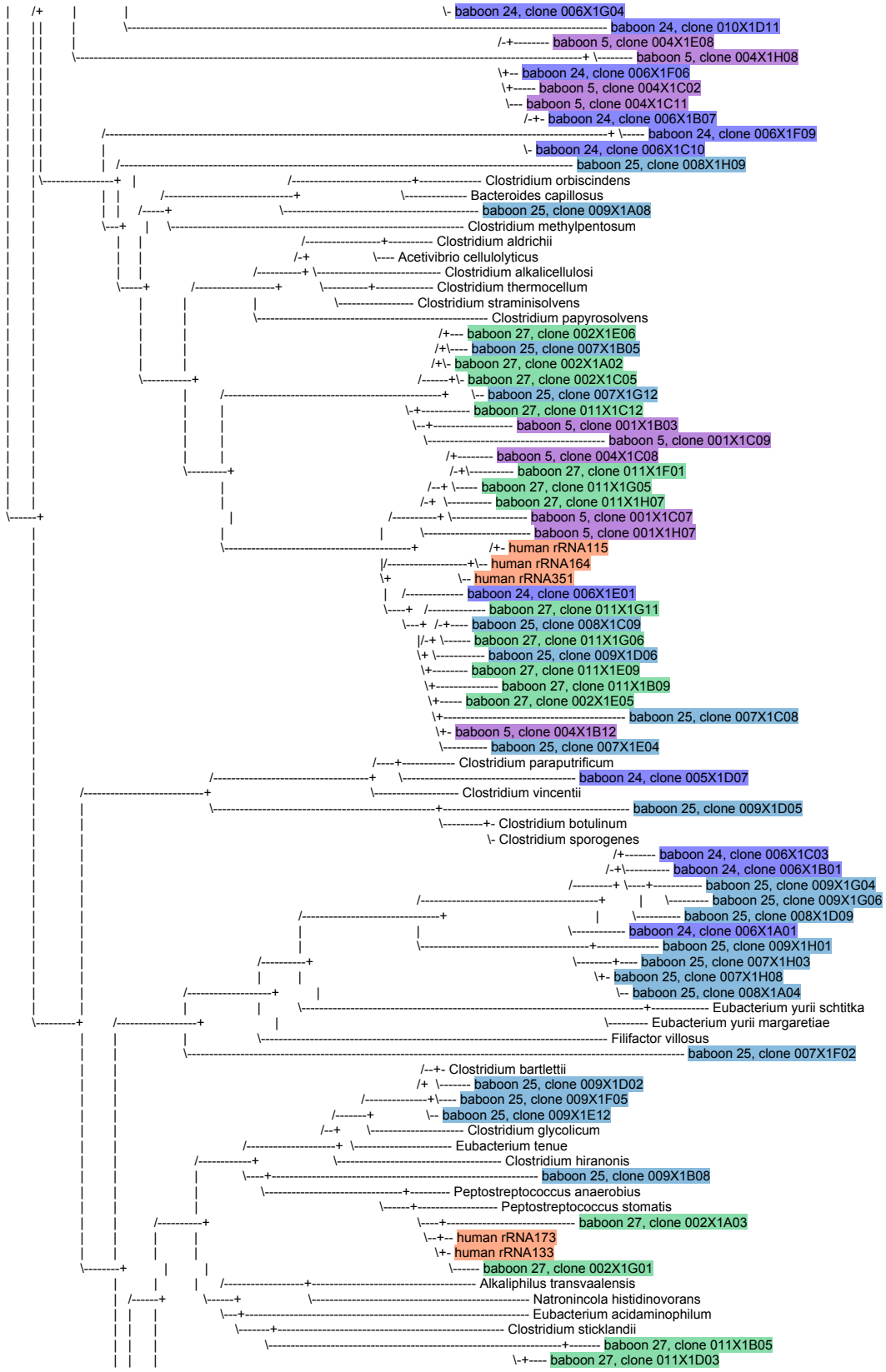

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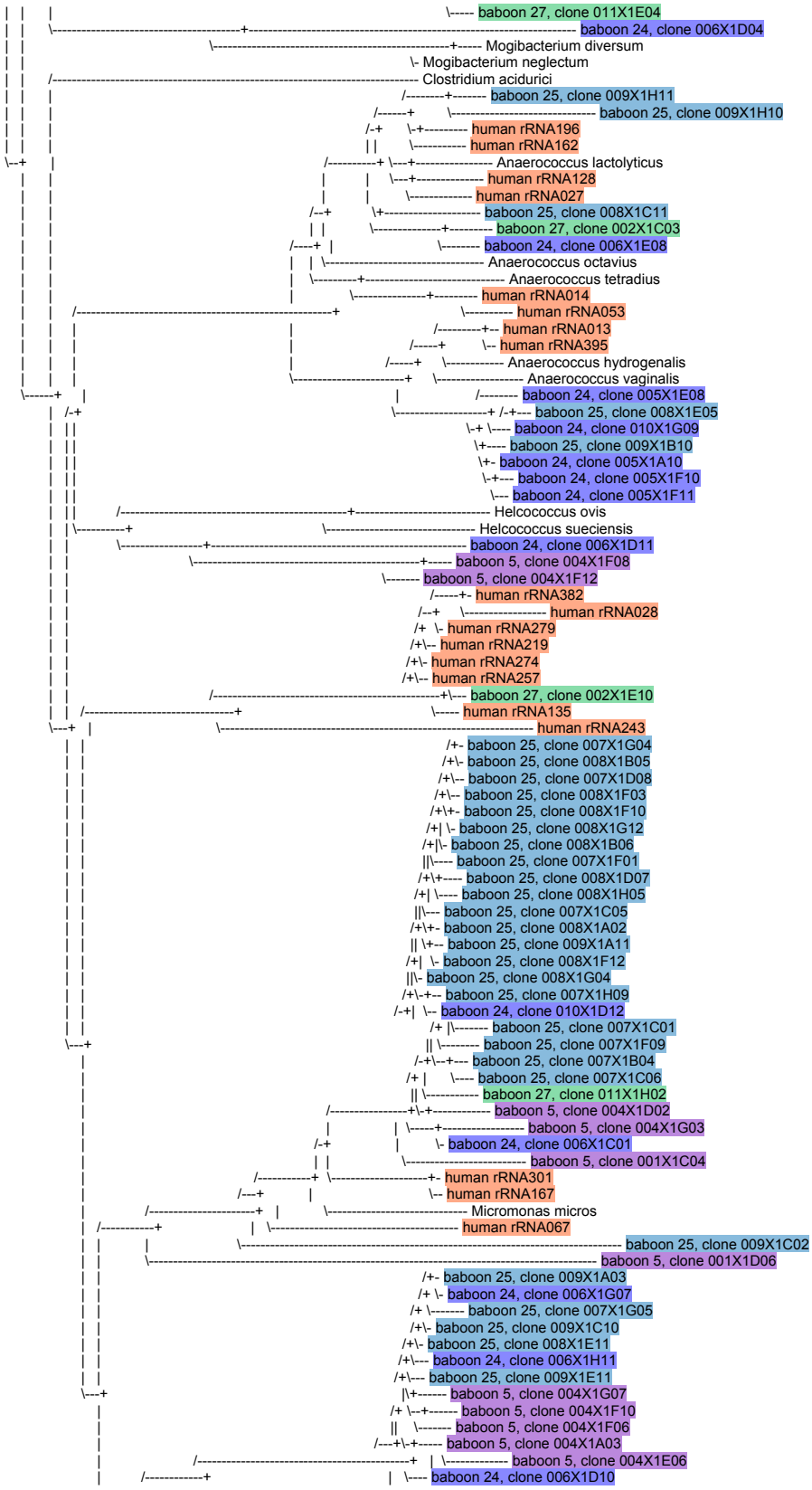
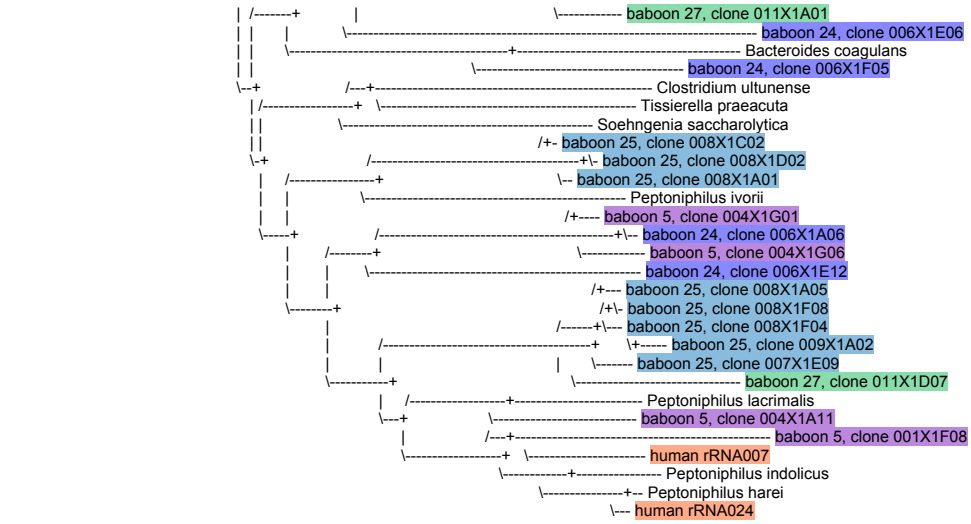
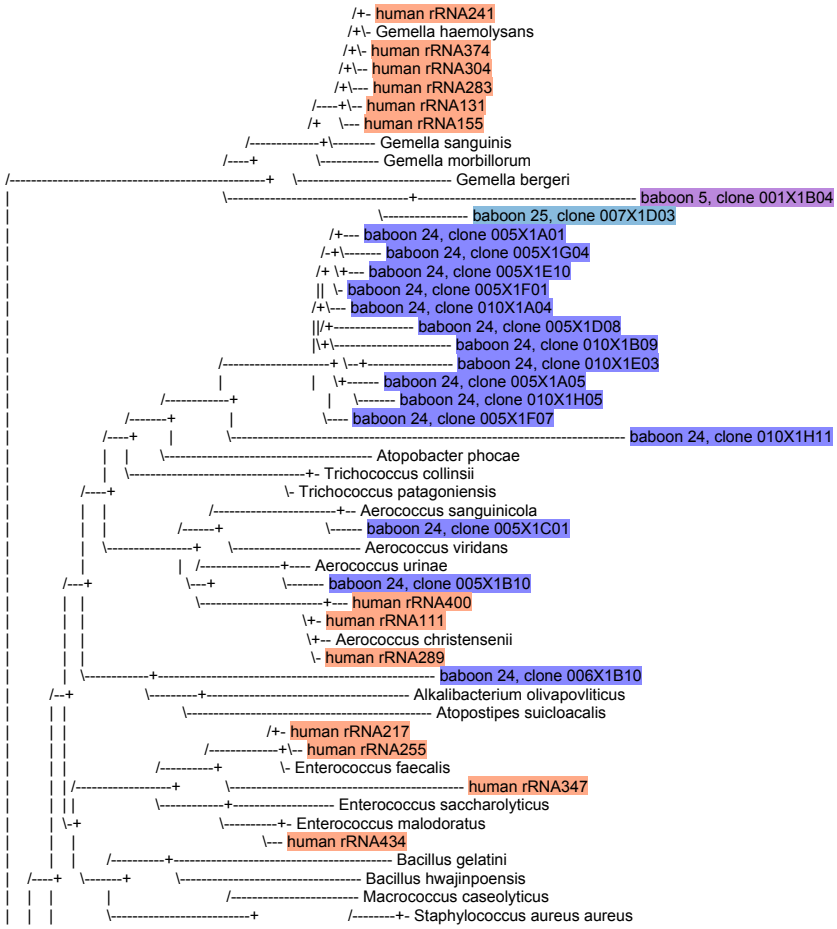


Figure 3.5 (cont.)



(B) Bacilli



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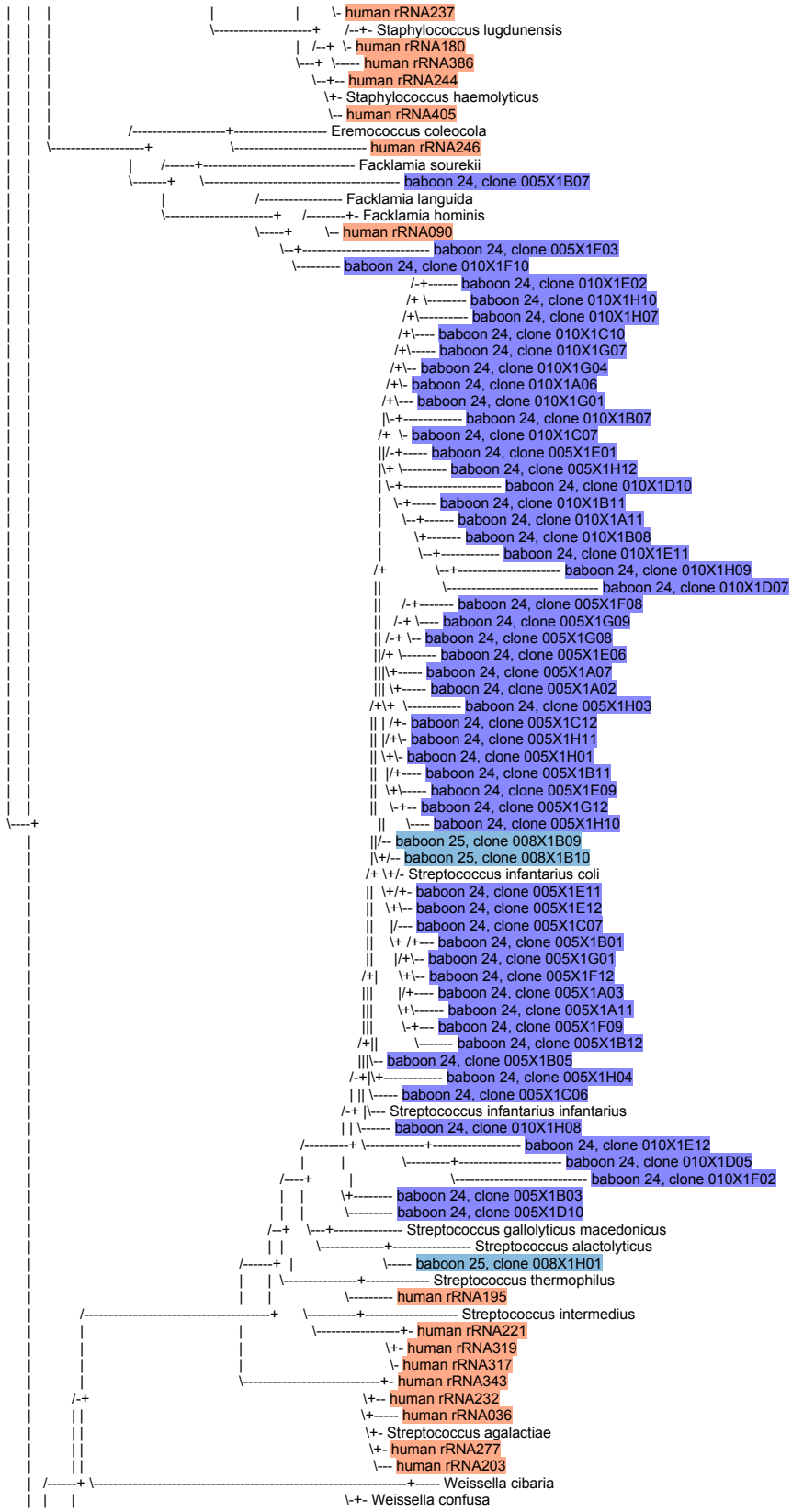

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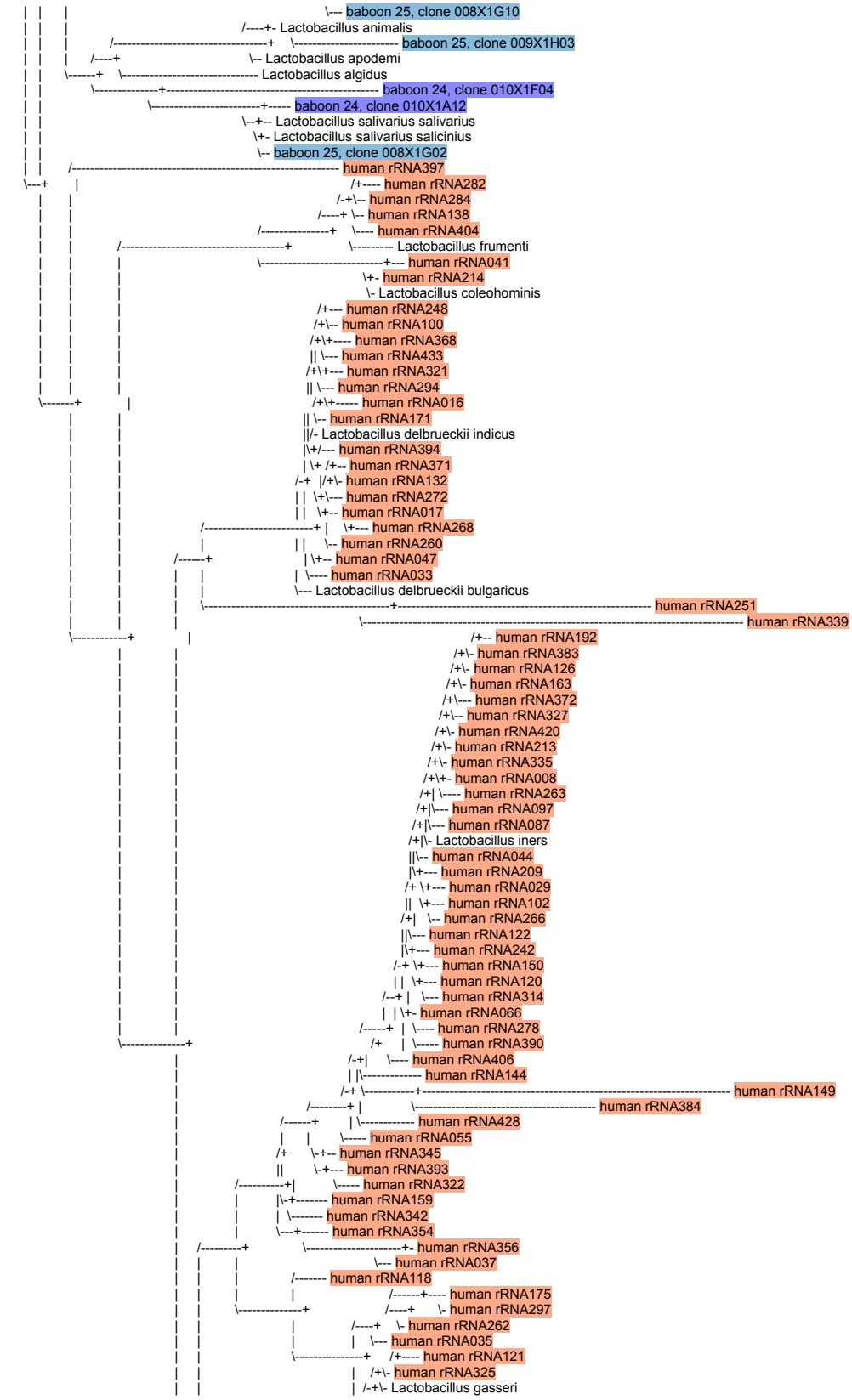

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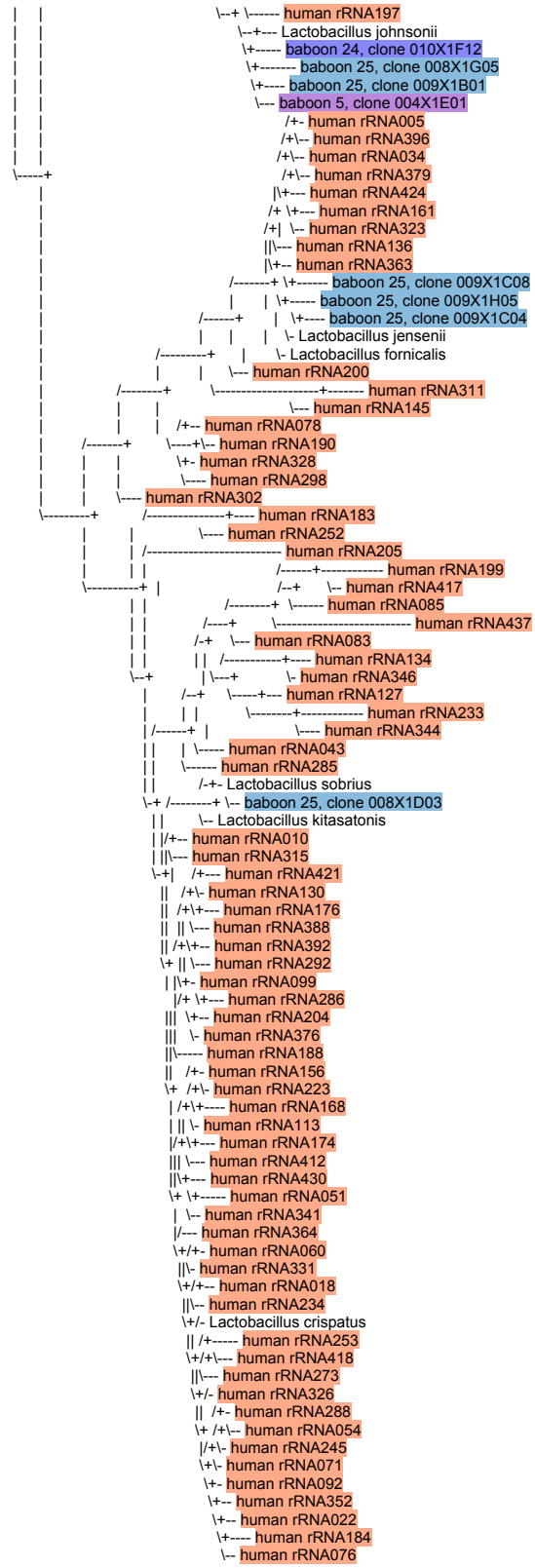

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(C) Mollicutes

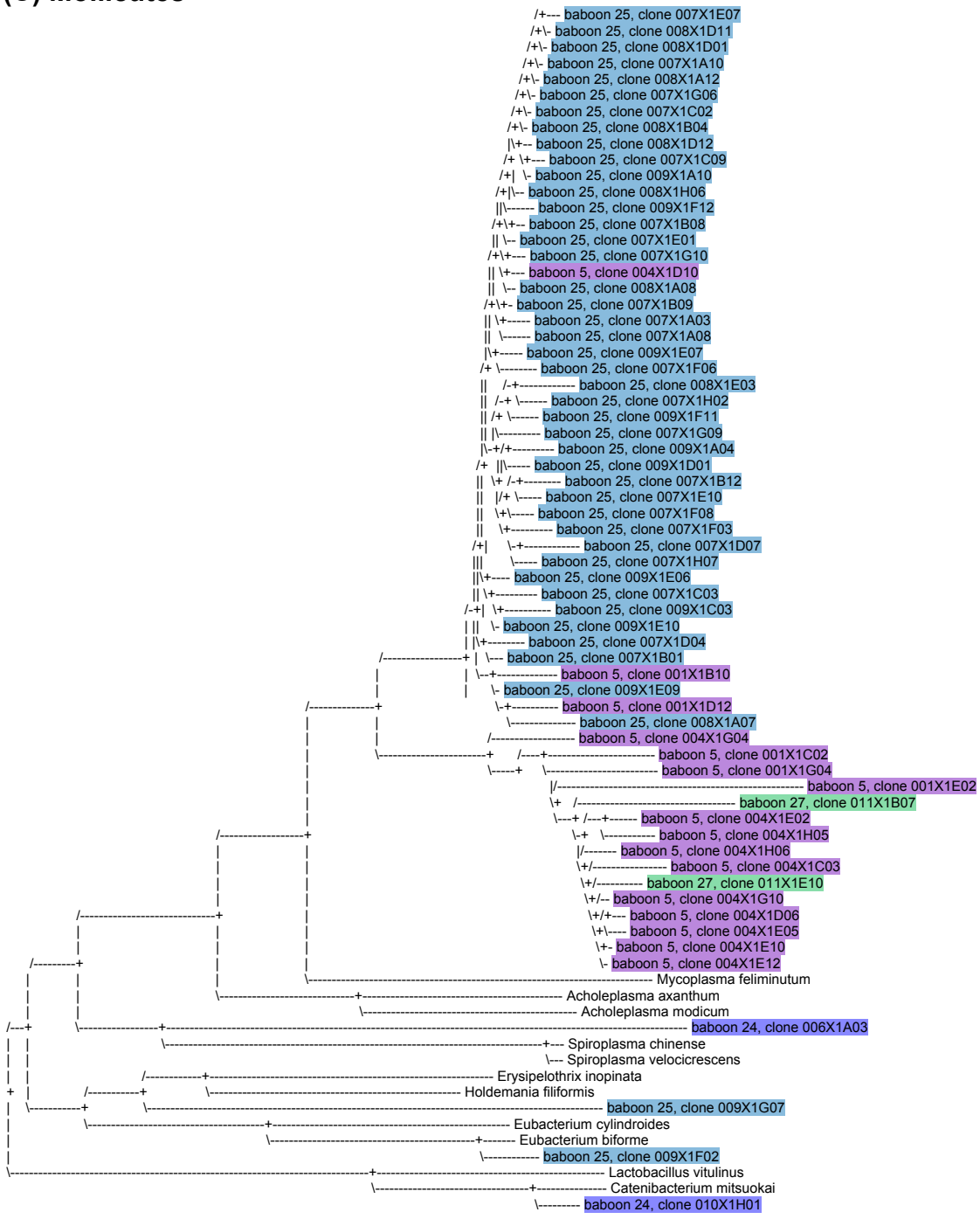
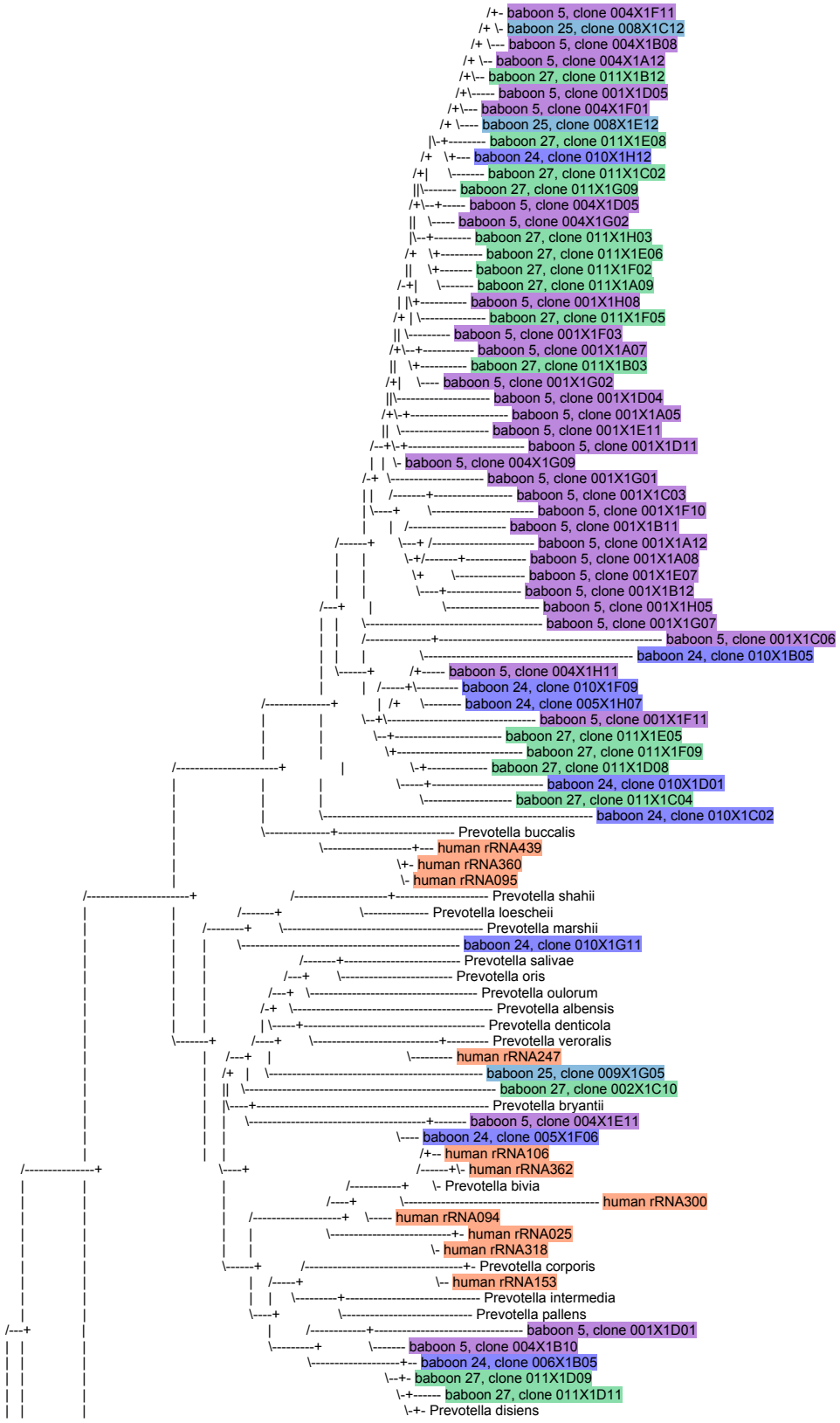


Figure 3.5 (cont.)

Figure 3.5 Phylogenetic relationships between sequences belonging to the Firmicutes. Three main classes were found within this phylum, Clostridia (**A**), Bacilli (**B**) and Mollicutes (**C**) with a particularly large diversity found within the Clostridia group. Clostridial sequences from baboon samples clustered in individual clades that diverged to higher than a 5% different from the human sequences. One exception was two clades, including close relatives to *Dialister* and *Megasphaera*, where human sequences clustered with baboon sequences. Taxa within the Bacilli class where derived from human samples primarily dominated by lactobacillus species. Close relatives of *Lactobacillus* species where found among the human-derived species, with some baboon-derived sequences clustering in this group. Representatives of the Mollicutes were found only in the baboon-derived sequences and approximately 94% of the sequences in this group were distant relatives of *Mycoplasma* species.

Figure 3.6 Bacteroidetes



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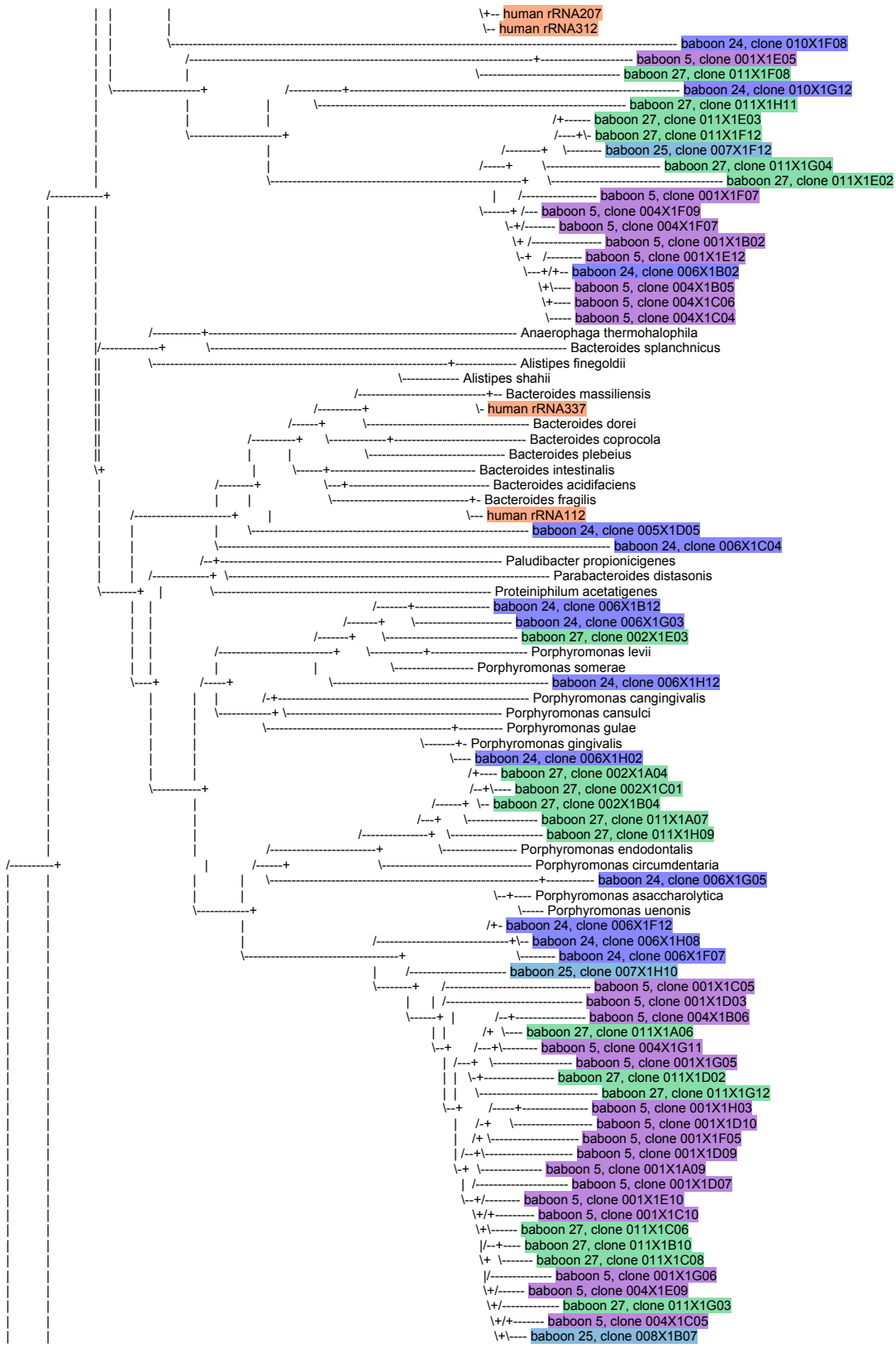

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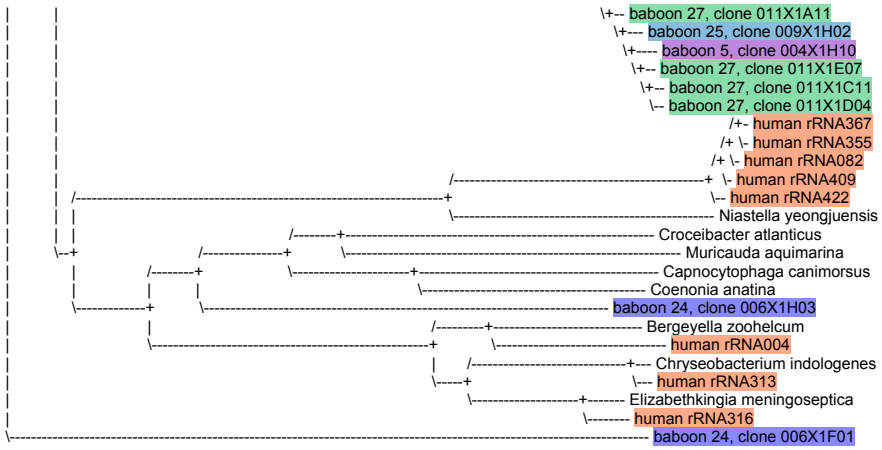
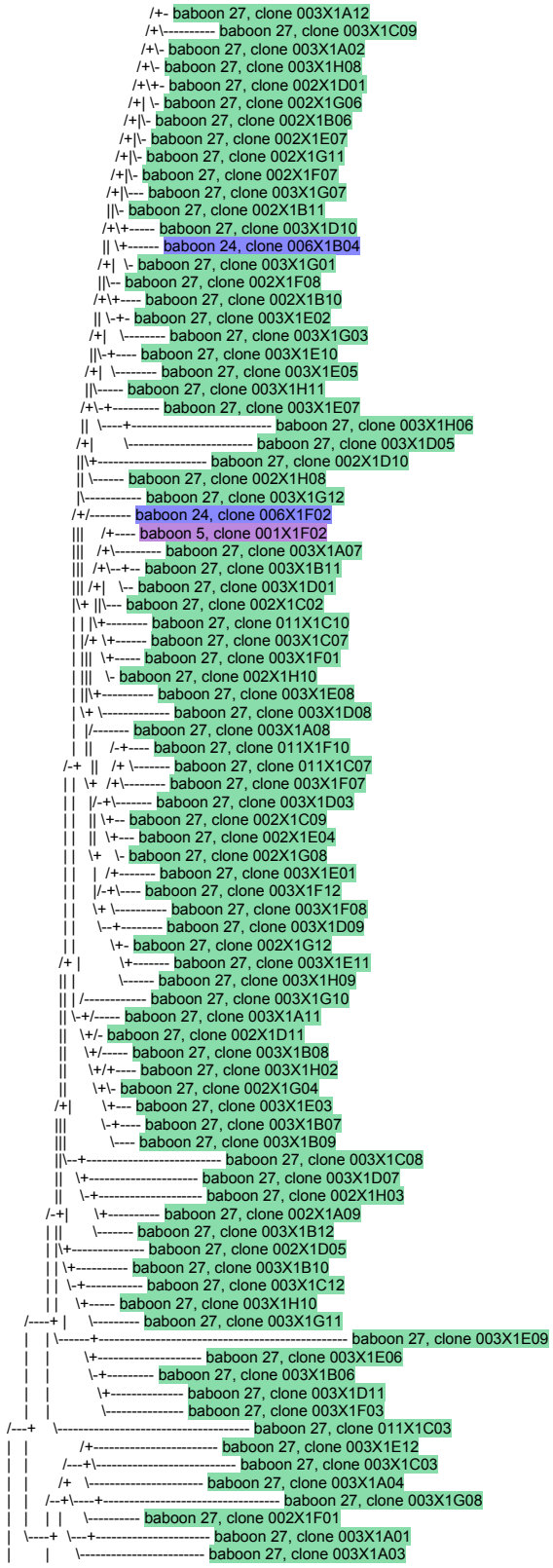


Figure 3.6 Phylogenetic relationships between rDNA sequences belonging to the Bacteroidetes. Baboon-derived sequences clustered in three large clades, consisting mainly of distant relatives of *Prevotella* and *Porphyromonas* species. Sequences derived from baboon and human microbes clustered in separate clades.

Figure 3.7 Fusobacteria



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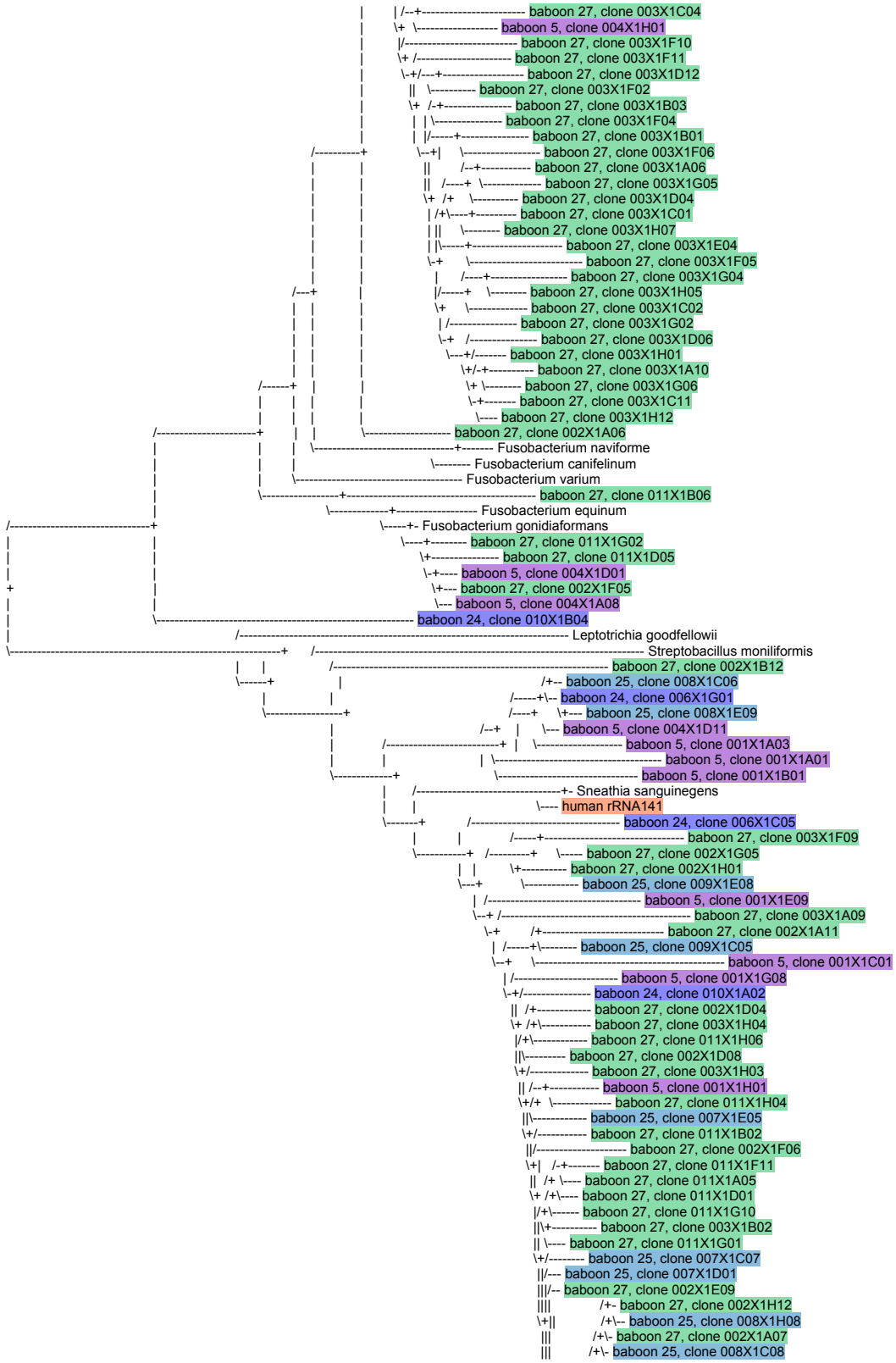

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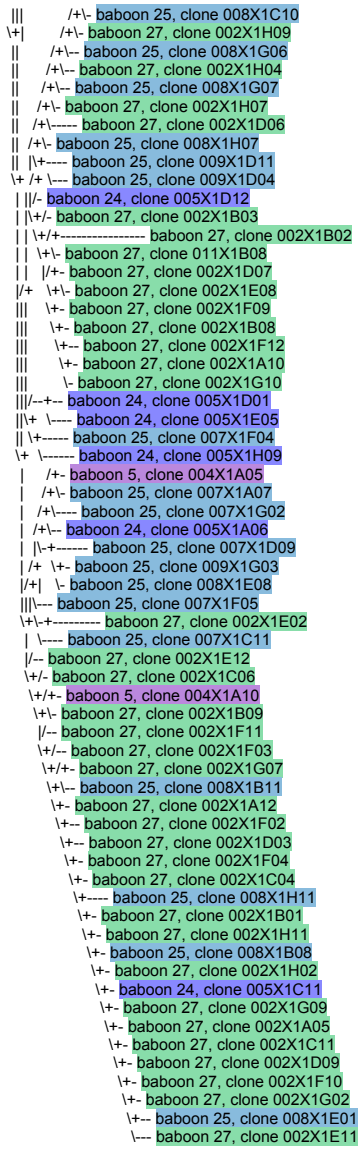


Figure 3.7 Phylogenetic relationships between rDNA sequences belonging to the Fusobacteria. Clusters of baboon-derived sequences were found to be phylogenetically related to species belonging to *Fusobacterium* and *Leptotrichia*. Baboon-derived sequences were most noticeably diverse within the *Fusobacterium*-related clades. Ribotypes found in baboon samples were dominant in this phylum, with the exception of one human clone found to be closely related to *Sneathia sanguinegens*.

Figure 3.8 Actinobacteria

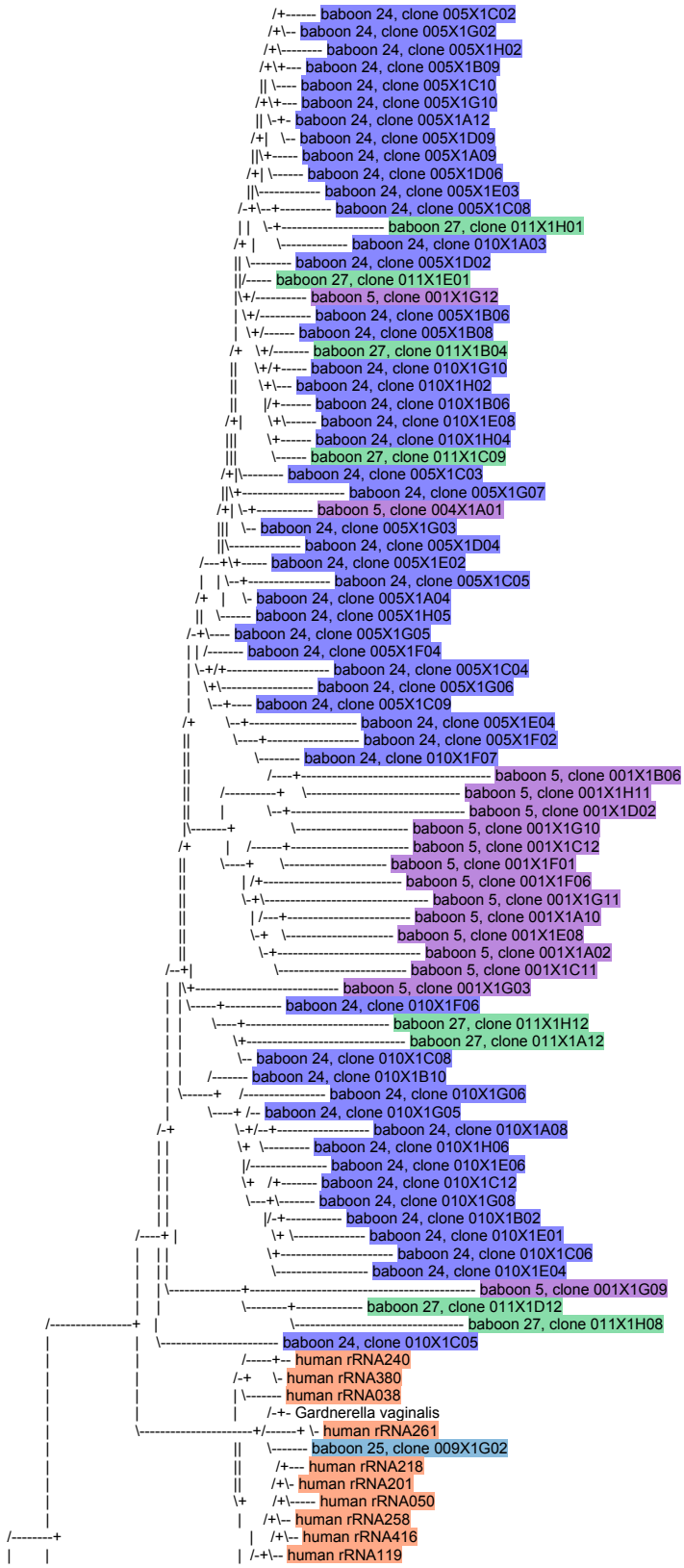


Figure 3.8 (cont.)

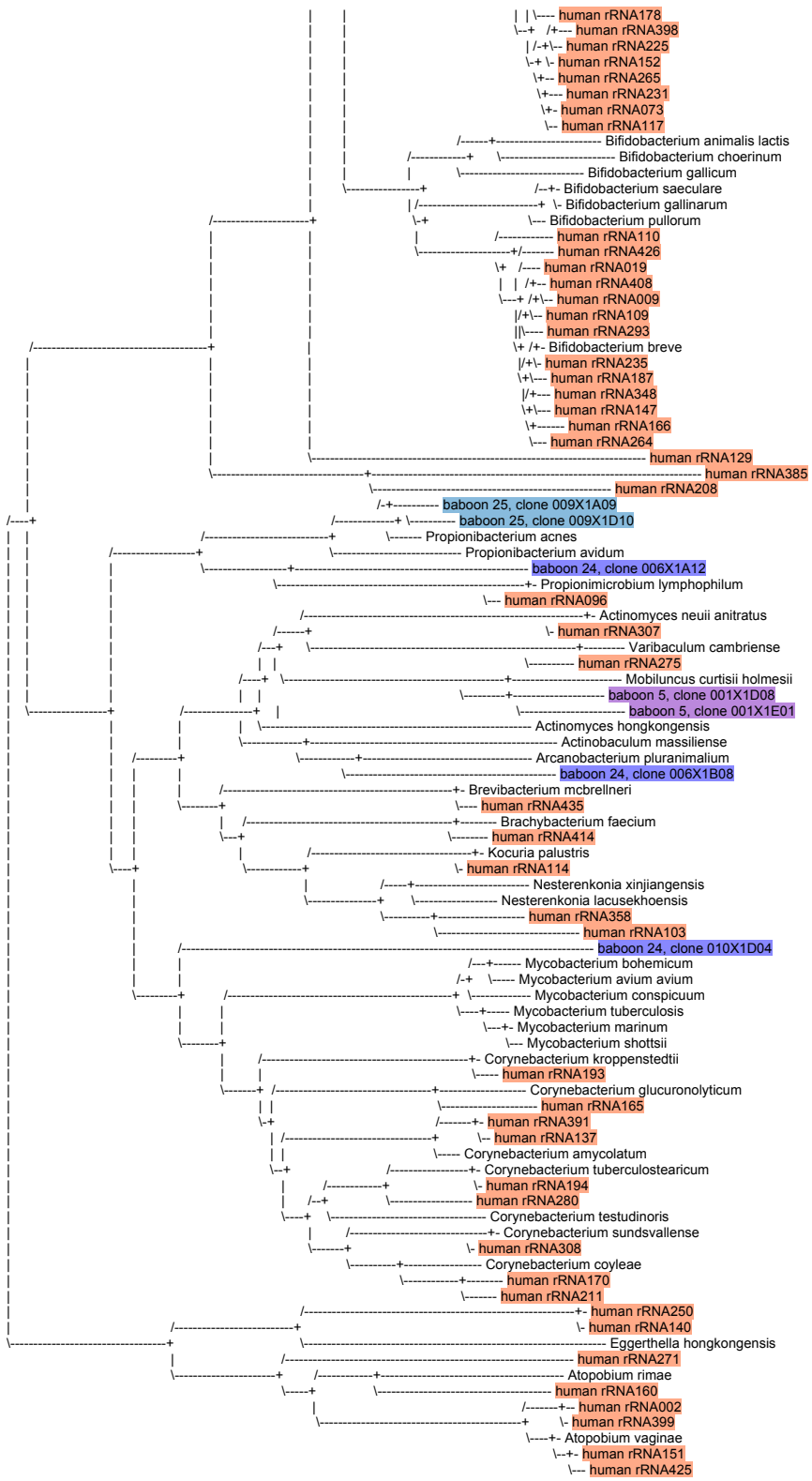


Figure 3.8 (cont.)

Figure 3.8 Phylogenetic relationships between rDNA sequences belonging to the Actinobacteria. Baboon-derived sequences were found to constitute 58% of taxa within Actinobacteria while humans represented 42%. Of the baboon sequences found in this group, 55% sequences clustered in a distinct clade that was most closely related to *Gardnerella vaginalis*. Human-derived sequences contained close relatives of *G. vaginalis* but significant divergence was observed between baboon and human sequences in this clade. A second cluster of human-derived sequences closely related to *Bifidobacterium* species. The remaining sequences for both baboon and human clustered in a distinct group. Some interdigitation was observed here, but no baboon sequences were closely related to human-derived sequences.

Figure 3.9 Spirochaetes

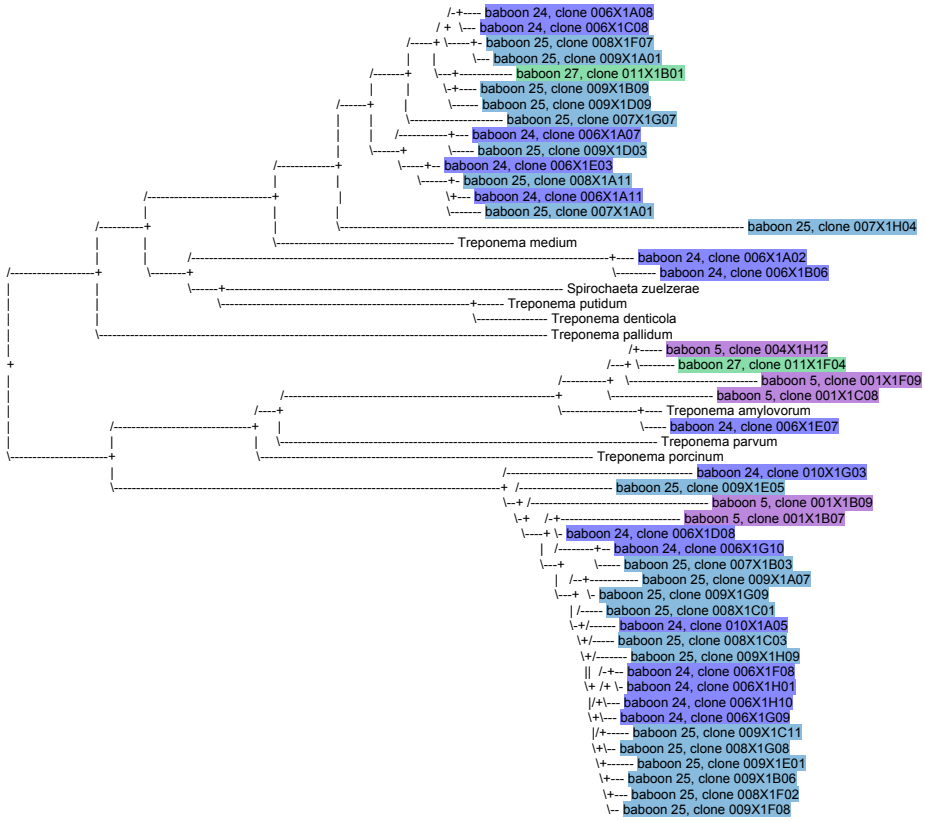


Figure 3.9 Phylogenetic relationships between rDNA sequences belonging to the Spirochaetes. A number of baboon-derived sequences clustered within the Spirochetes phylum. Most of these were related to *Treponema* species. This is apparent in a clade constituted of 9% of the sequences associated to this phylum to be closely related to *Treponema amylovorum*. No human-derived sequences were found to have any relationship to this group. Sequences outside this mentioned clade (91%) exhibited divergences greater than 10%, suggesting the presence of uncharacterized *Treponema* related species in the baboon microbial population analyzed in this study.

Figure 3.10 Proteobacteria

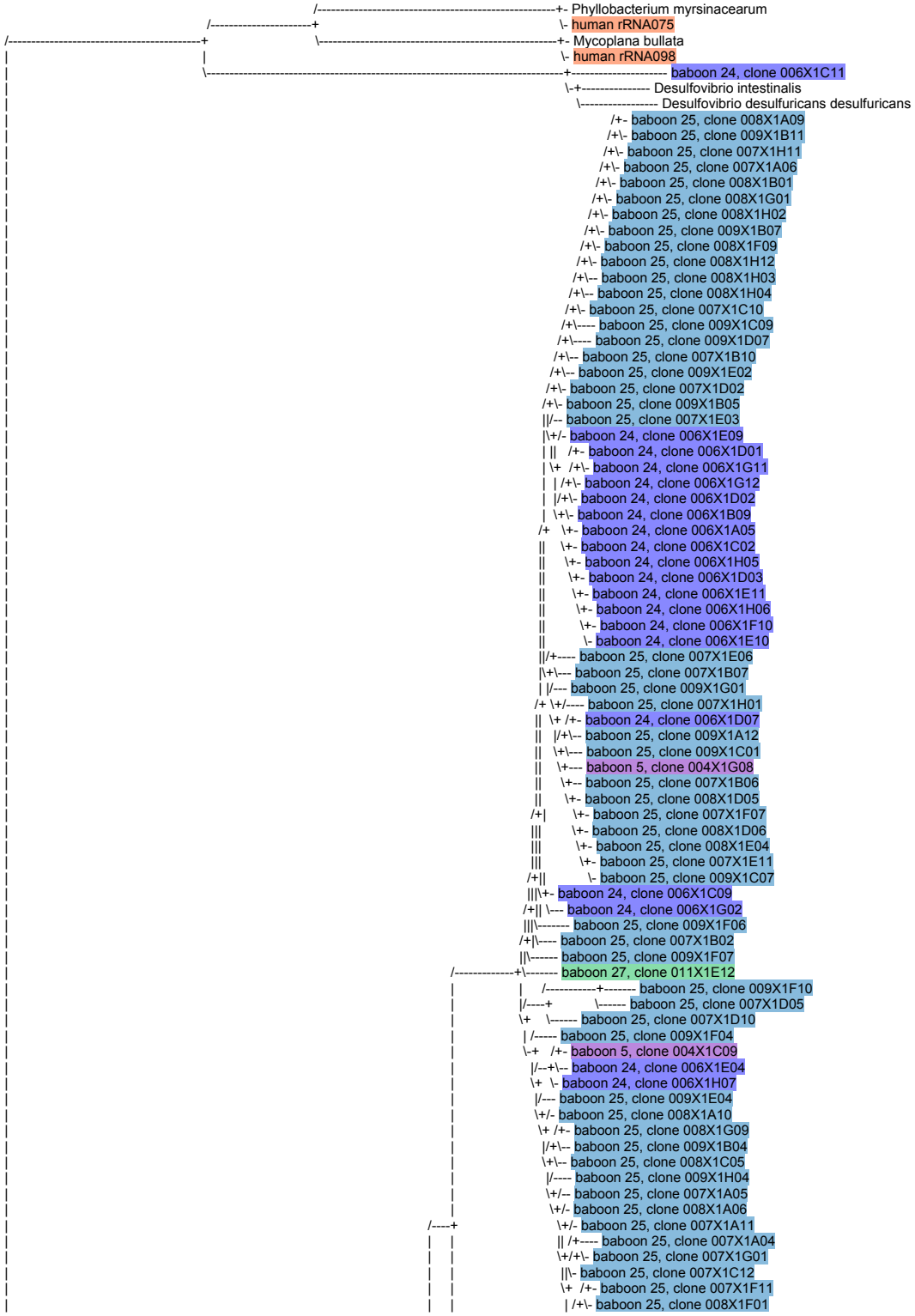


Figure 3.10 (cont.)

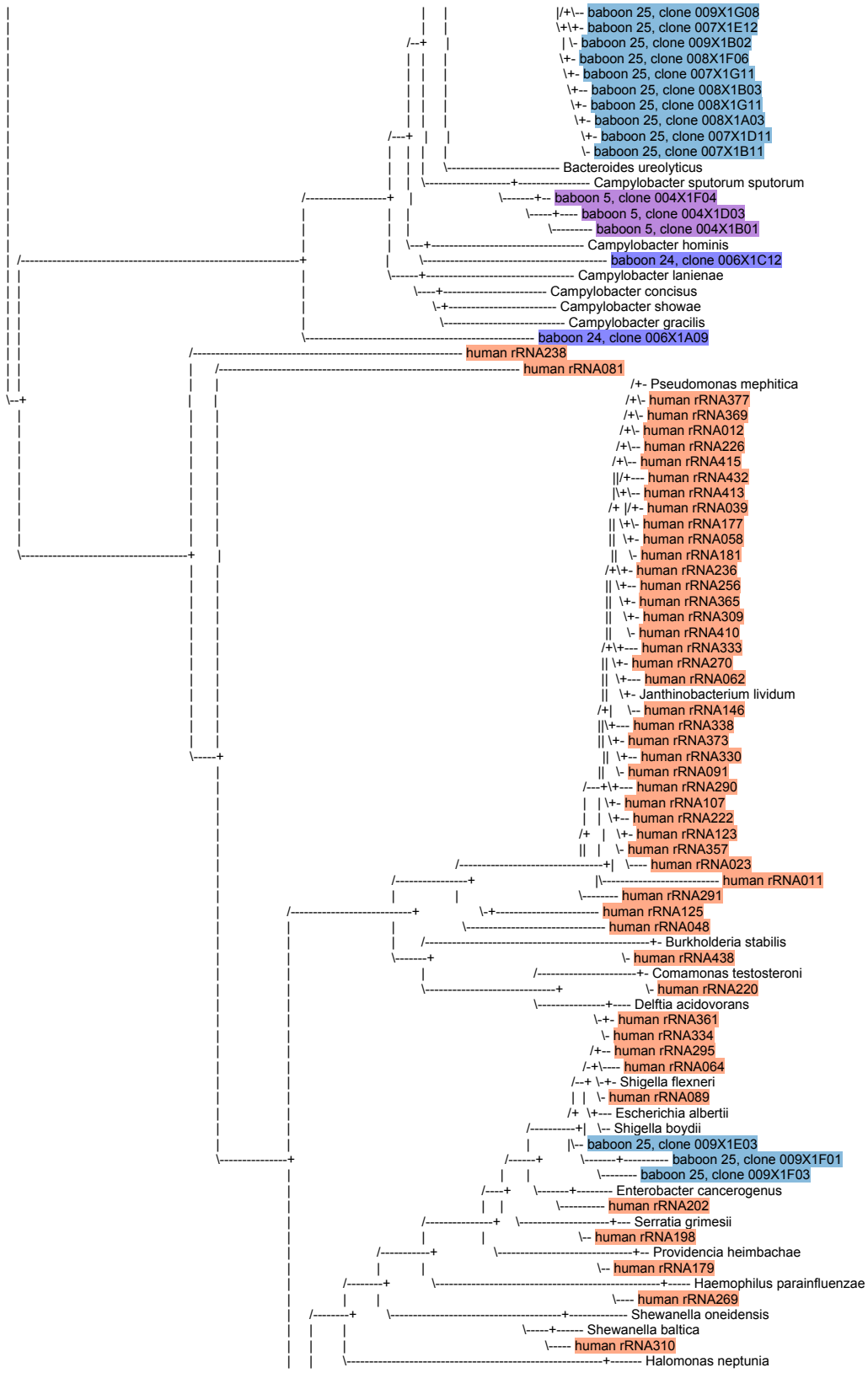


Figure 3.10 (cont.)

Figure 3.10 (cont.)

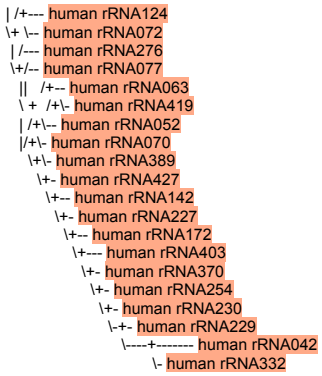


Figure 3.10 Phylogenetic relationships between rDNA sequences belonging to the Proteobacteria. Sequences associated with proteobacterial classes alpha, delta, gamma and epsilon were found. Alpha-proteobacteria consisted of only two human derived sequences closely related to species *Mycoplana* and *Phylobacterium*. A baboon-derived singleton of the Delta-proteobacteria was found to be a close relative of the genus Desulfovibrio. Gamma Proteobacteria representation consisting mainly (98%) of human derived bacterial sequences with clusters of closely related Pseudomonads and a region of greater diversity where 2% was represented by baboon derived sequences. Baboon sequences are close relatives of *Shigella* species. Interestingly a closely related cluster (1-2% divergence) of sequences which closer relationship to *Bacteroides ureolitycus* were the sole constituent of the Epsilon Proteobacteria group. *B. ureolitycus* is considered insertae sedis since it is genotypically related to the *Campylobacter* genus but cannot be classified as such based on proteolytic metabolism and fatty acid components (23).

CHAPTER 4

OTHER WORK

DIRECT VISUALIZATION OF PROLINE UTILIZATION REGULATORY COMPLEXES BY ATOMIC FORCE MICROSCOPY: THE PUTASOME

INTRODUCTION

Proline can be used as a sole carbon, nitrogen, and energy source by *Salmonella typhimurium*, *Escherichia coli* and other enteric bacteria (6). Catabolism of this amino acid requires the expression of two gene products located in an operon at 22 minutes on the *S. typhimurium* and *E. coli* chromosomes (6, 23, 35). The proline utilization (*put*) operon is an example of a system that is negatively regulated by a bifunctional (moonlight) enzyme. In *Salmonella,* these two genes share a 420 bp regulatory region that is located between them (15, 16). The first is the *putP* gene, which encodes an integral membrane protein with an apparent molecular weight of 25 kDa. This enzyme is the primary proline permease that catalyzes active transport of proline inside the cell by sodium-proline symport (5, 28). The second, PutA, is a moonlight protein with regulatory and enzymatic activities that shuttles between the membrane and the cytoplasm (22, 24, 35). When bound to the membrane PutA catalyzes the two-step oxidation of proline to glutamate. In the cytoplasm, specifically in the absence or low concentrations of proline, PutA represses its own transcription by negatively regulating of the *put* operon (23, 24).

Although regulation of *put* expression has similarities to other prokaryotic regulatory systems (34), there are some features that make regulation of the *put* operon unique. First, unlike the majority of the characterized repressors, PutA is a large protein with a monomeric molecular weight of 144 kDa. Second, as mentioned above, PutA functions both as a repressor and a bifunctional enzyme catalyzing the two-enzymatic steps involved in the oxidation of proline (24). Third, in order to be enzymatically active, PutA is released from the *put* operator DNA and becomes membrane associated (14, 23, 24). In order to carry out all these activities PutA must bind proline, membranes, FAD & NAD cofactors, P5C intermediates, and specific regions of DNA, (13). Therefore, regulation of proline utilization provides a model for peripherally membrane associated protein behavior that regulates gene expression in response to the presence or absence of substrate, in this case proline and available membrane sites.

Proline utilization mechanism

In the absence of proline, PutA accumulates in the cytoplasm and functions as an autogenous repressor, binding multiple operator sites in the *put* control region to repress transcription from *putA* and *putP* (Figure 4.1) (13, 24, 34). Induction of the *put* operon *in vivo* requires proline and available membrane sites (27, 40). Proline binding to PutA causes a change in the redox-state and the conformation of the protein stimulating association with the membrane (27, 33). When membrane associated, PutA is a bifunctional dehydrogenase with both proline dehydrogenase and pyrroline-5-carboxylate dehydrogenase activities,

catalyzing the two-enzymatic steps involved in the catabolism of proline (2, 24). During the first step, proline dehydrogenase catalyzes oxidation of proline to pyrroline-5-carboxylate (P5C). This reaction is coupled with the reduction of a tightly associated FAD cofactor (2, 24). The electrons from the reduced FAD are directly transferred to the electron transport chain in the cytoplasmic membrane (1, 2). Because the FAD cofactor is tightly associated with PutA, membrane association is absolutely required for reoxidation of PutA protein *in vivo* (1, 2, 43). During the second enzymatic step P5C dehydrogenase couples oxidation of P5C to glutamate with the reduction of soluble NAD (Figure 4.1) (2). Thus, induction of the *put* operon by proline involves a change in the cellular localization of PutA from the cytoplasm to the membrane (23, 24, 43). Additional studies also shown that proline dehydrogenase activity is required for induction of the *put* operon (26). Analysis of PutA super-repressor ($PutA^S$) mutants showed no increase in DNA affinity. However, these mutants affected the V_{max} or $Km_{(PRO)}$ for proline dehydrogenase activity. Amino acid substitutions in these $PutA^S$ mutants simultaneously affected induction by proline and proline dehydrogenase activity (26). These results imply that a single function is involved in both induction and proline dehydrogenase activity.

Supporting evidence of the *put* operon regulation

Several lines of genetic evidence first indicated that PutA negatively regulates expression of the *put* genes (24). Null mutations in the *putA* gene (transposon insertions, deletions, and frameshift mutations) cause complete loss

of enzymatic and regulatory activities. Such mutations are designated as (A^-C^-), where A^- indicates loss of enzymatic activity and C^- loss of regulatory control. There are three classes of *putA* missense mutants: *putA* (A^-C^-), which show the same phenotype as the null mutants; *putA* (A^-C^+), which lose enzymatic activity but retain regulatory control; and *putA* (A^+C^-), which retain the enzymatic properties but lose the regulatory control. These three classes of mutations map throughout the *putA* gene, indicating that both enzymatic and regulatory functions are located in a single polypeptide (24). Moreover, haploid *putA::lacZ* operon fusions express ß-galactosidase constitutively and regulation by proline is restored when wild-type PutA protein is provided in trans, (23). These results strongly suggest that PutA negatively regulates expression of the *put* operon. Electrophoretic Mobility Shift Assays (EMSA) demonstrated that PutA protein specifically binds to *put* control DNA *in vitro* (13, 34).

The *put* control region was identified by the isolation of promoter up mutations and operator constitutive mutations that affect *put* expression (15). The *put* regulatory region comprises the DNA between the *put* structural genes. DNA sequence analyses as well as in vivo and in vitro experiments indicated that this region is 420bp and that it contains multiple regulatory elements (15, 16, 32, 34) (Figure 4.2). Gel retardation assays showed that PutA specifically binds the *put* control region (34). PutA did not bind DNA lacking the control region, including ØX174, lambda DNA, or pBR322) (34). Methylation protection assays showed that PutA binds *put* control region DNA *in vivo* and that proline disrupts this interaction (33). In a $putA^+$ strain the *put* binding sites are sensitive to methylation

in the presence of proline but resistant to methylation in its absence. In a PutA^{S} mutant the *put* operators are protected from methylation in the presence or absence of proline. In *putA* ($A^{+}C^{-}$) mutants the *put* operators are sensitive to methylation in the presence or absence of proline (33). In addition, analysis of *put* expression on deletion mutants indicates that deletions that removed some of the potential operators show a higher basal level of *put* expression (33). However, these deletion mutants can still be induced by proline, indicating that they partially repress the *put* operon.

PutA cooperative binding

Cooperative binding to the operator sites has been suggested by previous studies, which indicated that the *put* control region has substantial intrinsic curvature (34) and IHF binding sites (32). Electrophoretic analysis of PCR products from the *put* control region showed altered mobility due to an intrinsic curvature in the DNA (34). Circular permutation assays confirmed the presence of a curved track of DNA in the *put* control region (34). This feature may be involved in the regulation of the *put* operon.

In the absence of proline, PutA accumulates in the cytoplasm where it is able to bind multiple operator sites in the *put* regulatory region. The presence of intrinsically curved DNA may promote interactions between PutA protein molecules bound to distant operator sites. Evidence of binding sites for Integration Host Factor (IHF), a protein that bends DNA, and intrinsic DNA bends in the *put* control region suggest that a higher order structure may be formed to

facilitate repression of the *put* operon (Figure 4.4)(32). The presence of IHF binding sites also suggests that a higher order structure may be involved in the repression of the *put* operon (32). IHF is a 20 KDa histone-like protein. It is composed of two different subunits, encoded by the *ihfA* and *ihfB* genes (8, 29, 30). IHF has the ability to wrap DNA on its surface resulting in a strong bend in the DNA (8). IHF plays an essential role in a variety of processes in *E. coli* such as packaging of DNA into viral capsids (18, 44), lambda site-specific recombination (7, 10, 38), initiation of plasmid replication, movement of transposable elements (25), and control of gene expression (9, 11, 12). IHF binds to specific consensus sequences and exerts its effect by bending the DNA surrounding these sites (45). For example, the effect of IHF in the site-specific recombination of lambda phage has been extensively studied (10, 14, 20). Mutations in the *ihfA* and *ihfB* genes prevent lambda site-specific recombination (37). The lambda Integrase protein (Int) and IHF bind cooperatively to the phage attachment site, *attP* (36). IHF bends the DNA at the *attP* site allowing Int protein bound at two different sites to interact forming a higher order structure called intasome (36, 37, 41). Similarly, when it is overproduced, binding of IHF to the *put* control region facilitates repression of the uninduced *put* operon *in vivo* (32). There are four consensus IHF binding sites in the *put* control region; however, gel shift assays and footprinting experiments showed that IHF only binds to two of those sites *in vitro* (Figure 4.2). The affinity of IHF for the binding sites in the *put* regulatory region is roughly equivalent to the affinity of IHF for the lambda H' site (21, 31, 32). IHF may induce the formation of a loop in the *put* control region

that facilitates repression of the *put* genes. These interactions may create a higher order structure necessary for complete repression of the *put* operon. This structure could be analogous to the "intasome", complex formed when lambda bacteriophage DNA integrate into the infected bacterial chromosome. Biochemical tools have provided hints for the existence of such a higher order structure, but there is no direct evidence for a protein-DNA complex. Evidence for this structure could arise by direct visualization coupled with analysis of mutants that alter the structure.

There are examples of other membrane proteins involved in genetic regulation, but most of these involve proteins that are part of two component regulatory systems. An integral membrane protein senses a signal in the periplasm and transmits it to a cytoplasmic regulatory protein, which modulates gene expression accordingly. In contrast, examples of membrane proteins that can directly affect gene expression are relatively rare. The proline utilization operon provides a model of how a peripherally associated membrane protein (PutA) can also regulate gene expression by changing its cellular localization.

The majority of the repressors that have been characterized are very small proteins. For example, the Lac repressor is 38 kDa and the TrpR repressor is 12 kDa. In contrast, PutA repressor is 144 kDa. In addition, many of the regulatory proteins that are extensively studied only show DNA binding activity. PutA is one of the few examples of a protein with both regulatory and enzymatic properties within the same polypeptide.

Operator region sites and PutA binding

In vitro evidence suggests that multiple operator sites in the *put* control region are necessary for complete repression of the *put* operon (13). Gel retardation assays were used to analyze binding of PutA to each of the potential operator sites *in vitro*. The effect of operator number on the affinity of PutA binding for the *put* control region was analyzed *in vitro* and *in vivo* (13). Findings showed that PutA binds *put* control region including only one potential operator very poorly (13). Also, PutA did not bind to DNA within the control region that lacks potential operator sites. An increase in the number of potential operators increases affinity of PutA for *put* control region DNA and its efficiency in repressing transcription (13). These studies show that PutA has a higher affinity when the whole *put* control region is present providing evidence for cooperative binding for the DNA-protein interaction. This may explain why the presence of multiple *put* binding sites is needed for the complete repression of the *put* operon (32, 34). Although we know that PutA is an autogenous repressor that binds to multiple operator sites in the *put* control region (23, 24, 34), little is known about the specificity geometric configuration of this DNA-protein interaction.

Atomic force microscopy (AFM)

The last 20 years have witnessed the extraordinary growth of structural studies in biology, and the impact is being felt in almost all areas of biological research (3, 4, 17). Several groups have used microscopy for the analysis of DNA, protein, and DNA–protein interactions. Until recently, electron microscopy was used as a

tool for imaging DNA, however this technique is harsh on biological samples, making successful analysis impossible (19). Several years ago scientists began to use Atomic Force Microscopy (AFM) for the analysis of biological samples. Successful characterization of biological specimens via AFM allowed the analysis of biological molecules faster, easier and more accurate. The development of the AFM and its introduction to imaging biological samples has provided scientists with a very powerful tool to explore many aspects of protein-protein, protein-DNA and many other interactions (4).

AFM modes of operation

AFM is a derivative of Scanning Probe Microscopy, which consists of a family of microscopy forms where a sharp probe is scanned across a surface and interactions between sample and the probe are monitored. AFM (also known as Scanning Force Microscopy or SFM) operates following the same general principles. In AFM a laser light is pointed off back of a cantilever where a sharp tip is located. The cantilever reflects the laser into a photodiode, which detects any cantilever deflection caused by tip sample interaction (Figure 4.3).
There are various modes of operation: Non-contact mode, contact mode, and tapping mode among others. However, tapping mode is the only method relevant to these studies. Tapping mode operates by scanning the tip attached to the end of an oscillating cantilever that travels across the sample surface. In this case, the cantilever is oscillated at near its resonance frequency with amplitudes ranging between 20 nm to 100 nm. The tip is positioned slightly above the

surface of the sample so that it only taps the surface for a very small fraction of its oscillation period. Tapping mode is the preferred mode of operation since it has features that allow better quality imaging with little deleterious effects on the sample. Analysis of samples in such mode provides higher lateral resolutions, which is critical when analyzing protein-DNA complexes. Lower forces applied with this mode cause less damage to soft samples and scraping is virtually eliminated since there are no lateral forces exerted on the sample, which makes it suitable for DNA structural analysis.

I have studied protein-DNA and protein-protein interactions involved in the regulation of the *put* operon via the formation of the PutAsome by directly visualizing these interactions using AFM imaging analysis. In this chapter I show images that illustrate the PutAsome geometry and configuration as a nucleoprotein complex. Efforts were focus on understanding the structure and its physiological characteristics by visualizing protein-DNA interactions using Atomic Force Microscopy.

MATERIALS AND METHODS

DNA fragments amplification

The three linear (1030 bp, 1026 bp and 435 bp) DNA fragments were obtained from the plasmid pBlueconII/*Oput* (13) plasmid by PCR amplification as previously described (13). To ascertain DNA absorption and immobilization we check adsorbed plasmid DNA on the mica surface 30 seconds after the deposition of DNA molecules were amplified by the polymerase chain reaction

(PCR) using plasmid pBlueconII/*Oput* as a template. The PCR reaction mix contained 20 pmoles of each primer, deoxynucleoside triphosphates (dNTP's) to a final concentration of 100 μM each, 1x PCR buffer (20 mM Tris-HCl, pH8.4, 50 mM KCl), $MgCl_2$ to a final concentration of 1.5 mM and 1U Taq polymerase (Invitrogen, California) in a final volume of 100 μl. The PCR amplification program consisted of an initial denaturation step at 93°C for 3 min, followed by 30 amplification cycles consisting of a denaturation step at 93°C for 1 min, an annealing step at 45°C for 1 min, and a DNA elongation step at 72°C for 1 min. A final elongation step was performed at 72°C for 5 min. The PCR products were ethanol precipitated and resuspended in TE buffer (1 mM Tris, pH 8.0, 50 mM EDTA). The DNA fragments were electrophoresed in a 2.0% NuSieve GTG agarose gel (Amersham) in 1x TAE buffer. The DNA bands were cut out of the gel and purified either by Qiaquick gel extraction (for DNA fragments less than 100 bp) or by Qiaquick gel extraction (Qiagen).

Protein purification

Integration Host Factor (IHF) protein was a generous give of Dr. Jeff Gardner in the Department of Microbiology at the University of Illinois Urbana-Champaign. PutA protein was obtained from the *E. coli* strain EM1475 that carries the expression plasmid pPC113. This plasmid construct places a 6-His tag on the amino-terminal end of the PutA protein. Purified PutA protein was obtained by affinity chromatography as described with minor modifications. A 5 ml culture of the EM1475 was grown overnight in LB broth containing ampicillin.

A 2 ml aliquot was subcultured into a liter of the same medium and was grown to early exponential phase (70-75 Klett units). PutA expression was induced by addition of IPTG to a final concentration of 1 mM and the culture was incubated for an additional hour at 37°C. The cell extract was prepared in G buffer (70 mM Tris-HCl, pH 8.2, 20% (v/v) glycerol) as previously described [Ostrovsky de Spicer, 1993 #81]. The purification was carried out by subjecting several aliquots of the cell extract to affinity chromatography on a His Trap Metal Chelating column (Ni-NTA Spin, Qiagen) by gravity flow and eluted with 250 mM histidine and a subsequent step consisting of a fast protein liquid chromatography system (FPLC) (Pharmacia). His•Tag PutA protein was eluted with a linear gradient of L-histidine (0-250 mM) in GA buffer (70 mM Tris-HCl, pH8.2, 100 mM NaCl, 20% (v/v) glycerol) [Liao, 1997 #123]. The fractions containing PutA protein were dialyzed and concentrated as described [Liao, 1997 #123][Muro-Pastor, 1995 #78]. PutA protein was obtained at a concentration of 2 mg/ml as determined by a Bradford assay (Bio-Rad). The percent purity of PutA protein obtained by this purification method was typically 90%. PutA was stored in G buffer (20% glycerol, 70 mM Tris, pH 8.2) to maintain its stability. The regulatory and enzymatic properties of purified PutA protein were analyzed by *in vitro* proline dehydrogenase assays and DNA binding assays as described [Menzel, 1981 #77][Muro-Pastor, 1995 #78].

AFM sample preparation

DNA (2.5 µg/mL) was deposited on mica in buffer containing divalent cations (20 mM Tris-HCl pH 7.5, 6 mM MgCl2, 50 mM NaCl). After DNA deposition on mica, free molecules were removed by thorough rinsing with the reaction buffer (20 mM Tris-HCl pH 7.5, 3 mM MgCl2, 50mM NaCl), and a variable concentration of the divalent cation magnesium. DNA fragments (0.1 ng/mL) were incubated with IHF purified protein (2.0 ng/µl), PutA purified protein (1.0 ng/µl mM). Reaction was allowed to incubate for 1 minute at room temperature. Complex containing solution was diluted 1:10 and aliquots of 10 µl were deposited in freshly cleaved mica. Upon nucleoprotein molecules absorption, samples were rinse as described above. After this rinsing step, the mica surface was rinsed, dried with pure nitrogen gas, and analyzed by AFM.

AFM imaging

Imaging was performed in Tapping Mode with a Multimode AFM (Veeco Metrology, Santa Barbara, CA) operating with a Nanoscope IIIa controller. We used silicon cantilevers (Veeco Metrology) with resonance frequencies of about 300 kHz. All images were collected at a scan frequency of 1.5 Hz and a resolution of 512 X 512 pixels.

RESULTS

Persistent length of the *put* control region DNA

Control region containing plasmid pBlueconII/*Oput* (13), was use to determine suitable parameters for subsequent DNA imaging and identify basic features in the DNA sample (Figure 4.5). Plasmid DNA was observed as open circle molecules in the mica surface. Upon closer inspection of a single molecule DNA showed irregular patterns indicative of the polymer helical turn. Intrinsic bends located in positions 200 and 230 along the *put* control region DNA and IHF binding sites located at positions 43 and 332 have been reported (32, 33). These features were examined by generating DNA fragments that contain the whole put control region. Bended DNA was apparent for several of the DNA molecules absorbed to the mica substrate where a cumulative bending effect was observed as *put* intergenic DNA posses two intrinsic bend sites (Figure 4.6).

The theoretical values of a base pair on a Watson-Creek B-form double helix are 3.4 Å (0.34 nm) on its axial rise and 20 Å (2 nm) on the helix diameter (39). Analysis of the *put* control region-containing fragment agrees with these values. The persistent length for a 435 bp DNA fragment resulted in approximately 147 nm (432 bp) based upon measurements of several immobilized DNA samples (Figure 4.7A). A larger fragment was engineered in order to obtain a molecule with the *Oput* region position at the center. These molecules of persistent length were found to be approximately 349 nm (1027 bp), consistent with this fragment theoretical value of 1030 nm (Figure 4.7B). These results show the accuracy of

this technique and provide preliminary “naked” DNA lengths for comparison against nucleoprotein complex measurements.

Visualization of purified PutA protein

PutA is a 144kDa bifunctional protein (22), which binds DNA as a dimer. The Stokes radius of PutA dimers from *E. coli* has been determined to be approximately 7.1 nm, and the results indicate PutA protein has an elongated irregular shape (2). Initial sample deposition revealed a degree of non-specific binding of PutA to AFM mica substrate suggesting that PutA could be immobilized. Images of these samples were obtained after deposition of purified PutA protein on to freshly cleave mica (Figure 4.8). In soluble form, PutA exists as a dimer, therefore it is reasonable to hypothesize that the protein binds the substrate in its dimer form. PutA protein was observed as a uniform set of molecules with similar height (Figure 4.8). To determine PutA height, bearing analysis was performed on a universe of PutA molecules (Figure 8A). This analysis allows the determination of the highest frequency height observed across the entire sample. Panel A in figure 4.8 illustrates a histogram describing the height distribution of a large sample of deposited PutA. Results suggests that the observed molecule range from 7.5 to 8.5 nm. Moreover, the area analyzed (bearing area percent) reveal that the molecules observed in the images cover near 100% measure the 7.8 nm in height, falling within the range of the propose size for PutA. In figure 4.8 panel B, a section analysis confirms the results

described above where measurement of single PutA molecules agree with the 8nm approximate height for the protein.

AFM visualization of IHF-DNA complexes

The Integration Host Factor protein (IHF) is a fairly small protein in comparison to PutA. Based on this I have imaged IHF bound DNA to confirm that the protein binds before proceeding with full complex imaging. A 329 bp fragment containing all the *put* control region operators and IHF binding site 332 was produced from *Cla*I digestion of the 435 bp fragment. The IHF binding site 32 and the intrinsic bend at position 200 were eliminated. Measurement of these fragments resulted in a persistent length of approximately 109 nm, consistent with of the 111.86 nm calculated value. Figure 4.9 Illustrates a 329 bp fragment bound IHF. Complexes exhibit DNA binding and bending of the DNA by the IHF protein.

Image analysis of full PutAsome complexes

To determine protein–DNA complex formation, samples containing PutA-DNA were prepared for AFM conditions and gel shift assays were performed observing binding of PutA to the intergenic region DNA. Mobility shift assays indicated PutA binding at concentrations averaging 1 ng/µl (range from 0.32 to 1.28 ng/µl) of protein. Gel shifts confirmed nucleoprotein complex formation. To assess geometric topography changes in the complex. Deletions of sets of operators were used (Table 4.1). Deletions of operators O1-O3 and O40-O5

showed partial binding. Full control region was bound and deletion of all operators, as expected, yielded only unbound DNA. These samples were prepared in the same fashioned and then deposited on mica and AFM analysis was performed. In a sample environment for AFM only the control region containing all operators formed a complex (Table 4.1).

Images of PutA-DNA complexes showed formation of a triangular protein arrangement (Figure 4.10A and B). These patterns suggest the possibility of a higher order structure formation. Surface plot images of these complexes were examined to determine protein size and DNA-Protein height differences in relation to the mica surface (Figure 4.10A). The analysis in panel B shows peaks representing proteins of approximately 7 nm in height, close to the 7.1 nm predicted PutA stoke radius by Brown & Wood. DNA width measurements resulted in a helical diameter of approximately 2 nm, consistent with the 20 Å theoretical values. Full PutAsome complexes (PutA-IHF-DNA) showed structures with similar features to the PutA-DNA complexes described above. An alpha structure is observed in these complexes with DNA measurements approximating 226 nm (666 bp) and peaks of 5 to 5.5 nm high. This data closely approximates to the theoretical values of a 691 bp fragment wrapping around a complex of proteins in the size range of PutA. The observed 25 bp size discrepancies in DNA measurements could be the result of protein bending of the DNA within that complex (Figure 4.11). Interestingly, when the 435 bp region was used for imaging, DNA arms were difficult to visualized suggesting complete sequestration of the DNA molecule (Figure 4.12). Surface plots of these

complexes do show an interesting feature of the PutA protein in complex. Upon high-resolution surface plot imaging of a single complex, the image revealed the contour of three molecules meeting at the center of the complex. This is consistent with the idea that PutA cooperativity may be driven by protein-protein interaction. To further support these findings, a 1030 bp fragment with the control region shifted to approximately 100 bp from the end on the DNA molecule was used in the formation of complexes. Protein-DNA images of these samples showed complex formation to the edge of the DNA molecule (Figure 4.13). The asymmetric structure served as a confirmation of the formation of this higher order complex. Our AFM data suggest the possibility of a higher order structures resembling the proposed PutAsome. Further studies are underway to confirm these results and further define these structures.

DISCUSSION

AFM imaging of DNA and proteins as static molecules on a surface is nowadays one of the standard tools in the analysis of DNA–protein complex formation. It has complemented numerous studies in order to elucidate molecular details of processes that play a role in different cellular machineries. AFM has been used to characterize the geometry and spatial relationship of several protein-nucleic acid complexes. I used this approach to confirm previous studies suggesting the formation of a higher order structure for the repression of the *put* operon. Current repression models based upon previous biochemical and genetic studies argued that the PutA mode of repression might hold certain

similarities to protein-DNA complexes formed in site-specific recombination events (e.g. Intasome). I have examined PutA-IHF-*Oput* DNA, “PutAsome” complexes by doing AFM imaging in air. Surface plots and section analysis of the images obtained support the existing evidence and adds real time data of the topological configuration of this complex (Figures 10, 11 &12).

The Protein-DNA interactions have being largely studied using gel electrophoresis-based assays. Such assays can be highly informative with regard to quantitating DNA bound protein, or determining sequence specificity using competitors. Also, results of electrophoretic mobility shift assays and footprinting techniques show average, or preponderance of protein-DNA interactions from a pool of molecular interactions.

AFM provides an alternative, complimentary method for studying the interactions of proteins with DNA in a more direct fashion. Dissection of the PutAsome by examining each component of the formed higher order structure separately was possible by way of AFM. For example, intrinsic bends on *Oput* DNA molecules could be observed prior to full complex examination (in Figure 4.6) allowing direct visualization of a feature only speculated upon previously by indirect data and modeling software (13, 34). Physiological details of the PutAsome are not apparent but suggestive when interpreting EMSA data. In Figure 4.10 we observed an alpha-like structure that suggest wrapping of the *Oput* molecule around a multimer of interacting PutA proteins. Consistent with this observation, persistent length measurements of naked DNA and in-complex DNA revealed that 95% of the control region is sequestered inside the loop

formed by the PutA and IHF proteins. This type of information can only be inferred with other methods. Here, I observed the phenomena directly. Interestingly, upon relocation (Shifting) of the control region along a larger fragment of DNA. One can observe formation of the PutAsome structures to the site where the intergenic region is located further supporting previous data (13). Topographical examination by high-resolution surface plot imaging, revealed three molecules interacting at the center of the PutAsome structure (Figure 4.12). This suggests the presence of interacting domains in the hydrophilic face of PutA that may contribute to the stabilization cooperative binding to the *put* operon regulatory region.

Taking all the data into account, one can suggest a model of repression where cumulative bending by the intrinsic curvature of the put control region DNA, in addition to bound IHF protein bring the control region to a conformation that allows for subsequent binding of PutA in a cooperative fashion (Figure 4.14). The alpha-like structure formed allows, potential protein-protein interactions that serve as a stabilizing factor for the tight repression of the proline utilization operon.

FUTURE WORK

Samples of PutA and IHF were prepared and deposited on mica for analysis. Nonspecific binding of PutA and IHF were avoided by forming complexes prior to deposition on mica. Nonspecific binding was not observed during analysis of these samples. However, in air imaging present numerous

challenges that prevent stepwise addition of constituents to observe PutAsome formation in different stages of its formation. One potential complication to this approach in AFM sample preparation relates to non-specific binding of proteins to the mica substrate. Mica is a SiAl mineral with perfect basal cleavage that provides an anatomically flat surface suitable for analysis of molecules in the nanometer range. However, it is important to consider that nonspecific binding of proteins to this substrate could bring complexity to the image interpretation. Proteins of interest that have absorbed to the mica substrate before complex formation due to greater affinity to the cleaved mica or any other binding issues can impede adequate analyses. A reasonable approached to resolve these issues constitute the implementation of a liquid cell to the AFM environment that will allow for a sounder atmosphere and adequate chemistries. Studies with dedicated AFM in which a chamber is filled with buffer and the tip is submerged to analyze protein and DNA molecules have been described (42). Techniques of this nature could allow me to perform more accurate analysis in a medium that more closely resembles PutA and IHF native environments. Similitude to the natural biochemical state of the nucleoprotein complex can present an opportunity to observe the formation of complex these structures in real time, revealing new mechanisms and adding valuable information to existing data. To fully understand the role dimer to monomer conversion plays in proline utilization regulation, and to provide a more accurate model for the molecular mechanism of repression, identification of domains in dimerization interface is critical and should be explore.

REFERENCES

1. **Abrahamson, J. L., L. G. Baker, J. T. Stephenson, and J. M. Wood.** 1983. Proline dehydrogenase from *Escherichia coli* K12. Properties of the membrane-associated enzyme. Eur J Biochem **134:**77-82.

2. **Brown, E. D., and J. M. Wood.** 1992. Redesign purification yields a fully functional PutA dimer from *Escherichia coli*. . The Journal of Biological Chemistry **267:**13086-13092.

3. **Bustamante, C., and C. Rivetti.** 1996. Visualizing protein-nucleic acid interactions on a large scale with the scanning force microscope. Annu Rev Biophys Biomol Struct **25:**395-429.

4. **Bustamante, C., J. Vesenka, C. L. Tang, W. Rees, M. Guthold, and R. Keller.** 1992. Circular DNA molecules imaged in air by scanning force microscopy. Biochemistry **31:**22-6.

5. **Cairney, J., C. F. Higgins, and I. R. Booth.** 1984. Proline uptake through the major transport system of *Salmonella typhimurium* is coupled to sodium ions. J Bacteriol **160:**22-7.

6. **Condamine, H.** 1971. Proline production regulation in *E. coli* K12. Ann Inst Pasteur **120:**126-43.

7. **Craig, N. L., and H. A. nash.** 1984. *E. coli* integration host factor binds to specific sites in DNA. Cell **39:**707-16.

8. **Flamm, E. L., and R. A. Weisberg.** 1985. Primary structure of the *hip* gene of *Escherichia coli* and of its product, the beta subunit of integration host factor. J Mol Biol **183:**117-28.

9. **Freundlich, M., N. Ramani, E. Mathew, A. Sirko, and P. Tsui.** 1992. The role of integration host factor in gene expression in *Escherichia coli*. Mol Microbiol **6:**2557-63.

10. **Gardner, J. F., and H. A. Nash.** 1986. Role of *E. coli* IHF protein in Lambda site-specific recombination. A mutational analysis of binding sites. J Mol Biol **191:**181-9.

11. **Giladi, H., M. Gottesman, and A. B. Oppenheim.** 1990. Integration host factor stimulates the phage lambda pL promoter. J Mol Biol **213:**109-21.

12. **Giladi, H., K. Igarashi, A. Ishihama, and A. B. Oppenheim.** 1992. Stimulation of the phage lambda pL promoter by integration host factor requires the carboxy terminus of the alpha-subunit of RNA polymerase. J Mol Biol **227:**985-90.

13. **Gomez-Curet, I.** 2001. Protein-DNA Interactions Required for Regulation of the Proline Utilization Operon in Salmonella. University of Illinois Urbana-Chamapaign, Urbana.

14. **Graham, S. B., J. T. Stephenson, and J. M. Wood** 1984. Proline dehydrogenase from *Escherichia coli* K12. Reconstitution of a functional membrane dehydrogenase association. J Biol Chem **259:**2656-61.

15. **Hahn, D. R., and S. R. Maloy.** 1986. Regulation of the put operon in *Salmonella typhimurium*: characterization of promoter and operator mutations. Genetics **114:**687-703.

16. **Hahn, D. R., R. S. Myers, C. R. Kent, and S. R. Maloy.** 1988. Regulation of proline utilization in *Salmonella typhimurium*: molecular characterization of the put operon, and DNA sequence of the put control region. Mol Gen Genet **213:**125-33.

17. **Hoyt, P. R., M. J. Doktycz, R. J. Warmack, and D. P. Allison.** 2001. Spin-column isolation of DNA-protein interactions from complex protein mixtures for AFM imaging. Ultramicroscopy **86:**139-43.

18. **Hwang, Y., and M. Feiss.** 1995. A defined system for in vitro lambda DNA packaging. Virology **211:**367-76.

19. **Le Cam, E., D. Frechon, M. Barray, A. Fourcade, and E. Delain.** 1994. Observation of binding and polymerization of Fur repressor onto operator-containing DNA with electron and atomic force microscopes. Proc Natl Acad Sci U S A **91:**11816-20.

20. **Lee, E. C., M. P. MacWilliams, R. I. Gumport, and J. F. Gardner.** 1991. Genetic analysis of *Escherichia coli* integration host factor interactions with its bacteriophage lambda H' recognition site. J Bacteriol **173:**609-17.

21. **MacWilliams, M., R. I. Gumport, and J. F. Gardner.** 1997. Mutational analysis of protein binding sites involved in formation of the bacteriophage lambda attL complex. J Bacteriol **179:**1059-67.

22. **Maloy, S. R.** 1987. The proline utilization operon. *In* F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, S. M., and U. H.E. (ed.), *Escherichia coli* and *Salmonella typhimurium*: cellular and molecular biology, 1st ed. American Society of Micrbiology, Washington in D.C.

23. **Maloy, S. R., and J. R. Roth.** 1983. Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put::Mu*d (*Ap, lac*) operon fusions. J Bacteriol **154:**561-8.

24. **Menzel, R., and J. Roth.** 1981. Regulation of the genes for proline utilization in *Salmonella typhimurium*: autogenous repression by the *putA* gene product. J Mol Biol **148:**21-44.

25. **Morisato, D., and N. Kleckner.** 1987. Tn10 transposition and circle formation in vitro. Cell **51:**101-11.

26. **Muro-Pastor, A. M., and S. Maloy.** 1995. Proline dehydrogenase activity of the transcriptional repressor PutA is required for induction of the put operon by proline. J Biol Chem **270:**9819-27.

27. **Muro-Pastor, A. M., P. Ostrovsky, and S. Maloy.** 1997. Regulation of gene expression by repressor localization: biochemical evidence that membrane and DNA binding by the PutA protein are mutually exclusive. J Bacteriol **179:**2788-91.

28. **Myers, R. S., D. Townsend, and S. Maloy.** 1991. Dissecting the molecular mechanism of ion-solute cotransport: substrate specificity mutations in the *putP* gene affect the kinetics of proline transport. J Membr Biol **121:**201-14.

29. **Nash, H. A., and C. A. Robertson.** 1981. Purification and properties of the *Escherichia coli* protein factor required for lambda integrative recombination. J Biol Chem **256:**9246-53.

30. **Nash, H. A., C. A. Robertson, E. Flamm, R. A. Weisberg, and H. I. Miller.** 1987. Overproduction of *Escherichia coli* integration host factor, a protein with nonidentical subunits. J Bacteriol **169:**4124-7.

31. **Nunes-Duby, S. E., L. I. Smith-Mungo, and A. Landy.** 1995. Single base-pair precision and structural rigidity in a small IHF-induced DNA loop. J Mol Biol **253:**228-42.

32. **O'Brien, K., G. Deno, P. Ostrovsky de Spicer, J. F. Gardner, and S. R. Maloy.** 1992. Integration host factor facilitates repression of the *put* operon in *Salmonella typhimurium*. Gene **118:**13-9.

33. **Ostrovsky de Spicer, P., and S. Maloy.** 1993. PutA protein, a membrane-associated flavin dehydrogenase, acts as a redox-dependent transcriptional regulator. Proc Natl Acad Sci U S A **90:**4295-8.

34. **Ostrovsky de Spicer, P., K. O'Brien, and S. Maloy.** 1991. Regulation of proline utilization in *Salmonella typhimurium*: a membrane-associated dehydrogenase binds DNA in vitro. J Bacteriol **173:**211-9.

35. **Ratzkin, B., and J. Roth.** 1978. Cluster of genes controlling proline degradation in *Salmonella typhimurium*. J Bacteriol **133:**744-54.

36. **Richet, E., P. Abcarian, and H. A. Nash.** 1986. The interaction of recombination proteins with supercoiled DNA: defining the role of supercoiling in lambda integrative recombination. Cell **46:**1011-21.

37. **Robertson, C. A., and H. A. Nash.** 1988. Bending of the bacteriophage lambda attachment site by *Escherichia coli* integration host factor. J Biol Chem **263:**3554-7.

38. **Segall, A. M., and H. A. Nash.** 1993. Synaptic intermediates in bacteriophage lambda site-specific recombination: integrase can align pairs of attachment sites. EMBO J **12:**4567-76.

39. **Sinden, R.** 1994. DNA Structure and Function. Academic Press.

40. **Surber, M.** 1999. Proline Utilization in *Salmonella typhimurium*: A Model for Regulation of Peripheral-Membrane Flavin Dehydrogenase-Lipid Interactions. Doctoral. University of Illinois Urbana-Chamnpaign, Urbana.

41. **Thompson, J. F., D. Waechter-Brulla, R. I. Gumport, J. F. Gardner, L. Moitoso de Vargas, and A. Landy.** 1986. Mutations in an integration host factor-binding site: effect on lambda site-specific recombination and regulatory implications. J Bacteriol **168:**1343-51.

42. **van Noort, S. J., K. O. van der Werf, A. P. Eker, C. Wyman, B. G. de Grooth, N. F. van Hulst, and J. Greve.** 1998. Direct visualization of dynamic protein-DNA interactions with a dedicated atomic force microscope. Biophys J **74:**2840-9.

43. **Wood, J. M.** 1987. Membrane association of proline dehydrogenase in *Escherichia coli* is redox dependent. Proc Natl Acad Sci U S A **84:**373-7.

44. **Xin, W., Z. H. Cai, and M. Feiss.** 1993. Function of IHF in lambda DNA packaging. II. Effects of mutations altering the IHF binding site and the intrinsic bend in *cosB* on lambda development. J Mol Biol **230:**505-15.

45. **Yang, C. C., and H. A. Nash.** 1989. The interaction of *E. coli* IHF protein with its specific binding sites. Cell **57:**869-80.

TABLES AND FIGURES

Oput DNA	EMSA	AFM
Wild type	+	+
Δ O1 – O3	+/-	-
Δ O4 – O5	+/-	-
Δ O1 – O5	-	-

+/- Represents partial binding observed in electrophoretic mobility shift assays of put control region DNA.

Table 4.1 Control region operator deletions. Deletions of set of operators were made and tested for their effect on the PutAsome formation by electrophoretic mobility assays and atomic force microscopy.

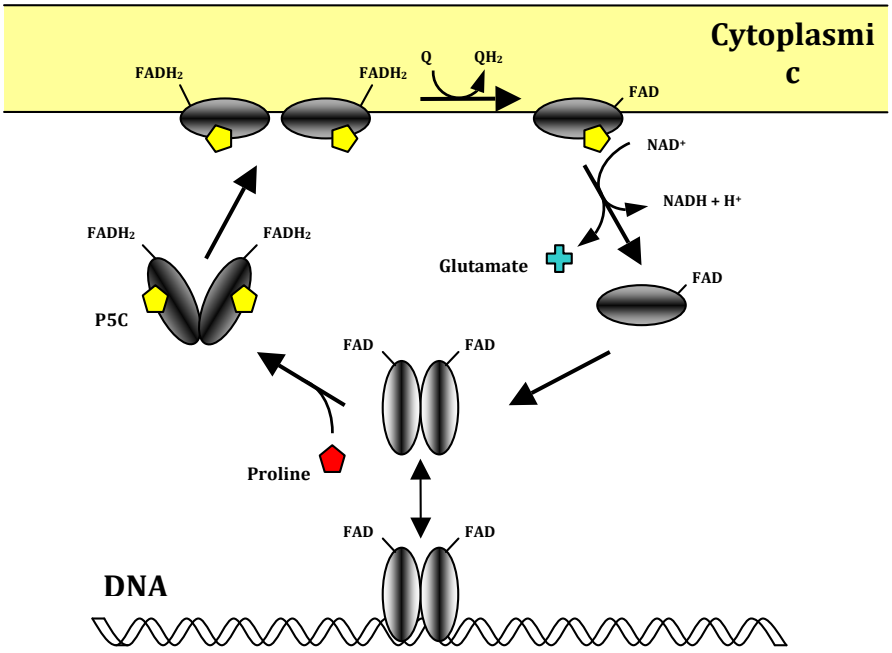


Figure 4.1 The Proline Utilization Model. At low intracellular proline, PutA is soluble and function as a transcriptional repressor of the *put* operon. As the concentration of proline increases, the FAD associated to PutA is reduced and proline is converted to Pyrroline 5 Carboxylate or P5C. These two factors alter the conformation of PutA and increase the hydrophobicity of the protein. After released from the DNA, PutA is driven to the inner leaflet of the cytoplasmic membrane where it functions as an enzyme. Both $FADH_2$ and P5C are required for maximal membrane association and the presence of P5C maintains the PutA-membrane interaction during the redox cycling of FAD. As proline levels decrease, both $FADH_2$ and P5C are oxidized, and PutA regains its original hydrophilic structure. At this point P5C have been converted to glutamate via a catalytic reaction coupled to the reduction of NAD. In this state PutA accumulates in the cytoplasm and becomes available to repress the *put* operon.

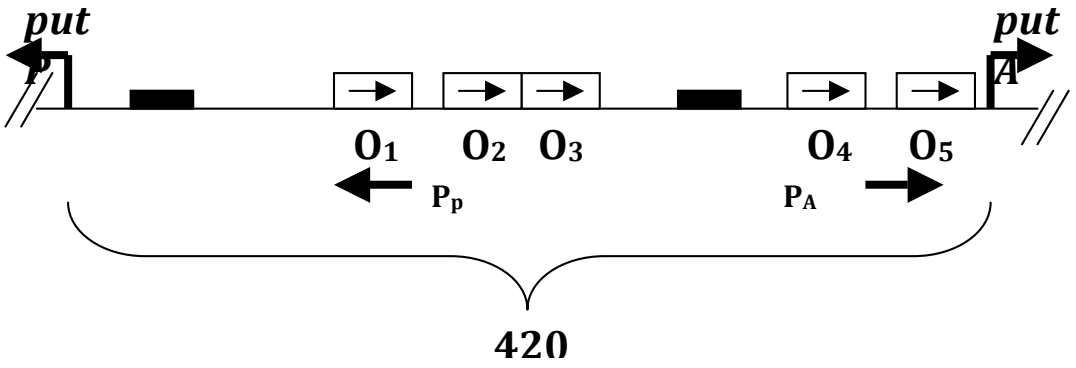


Figure 4.2 Representation of the *put* control region. *Oput* is flanked by the beginning of the *putA* and *putP* genes transactional start sites (shown with the bent arrows). The thick arrows represent the *putP* and *putA* promoters. Potential operator sites are shown in boxes with arrows that indicate the binding sites orientation. The shadowed rectangles indicate IHF binding sites along the *put* control region.

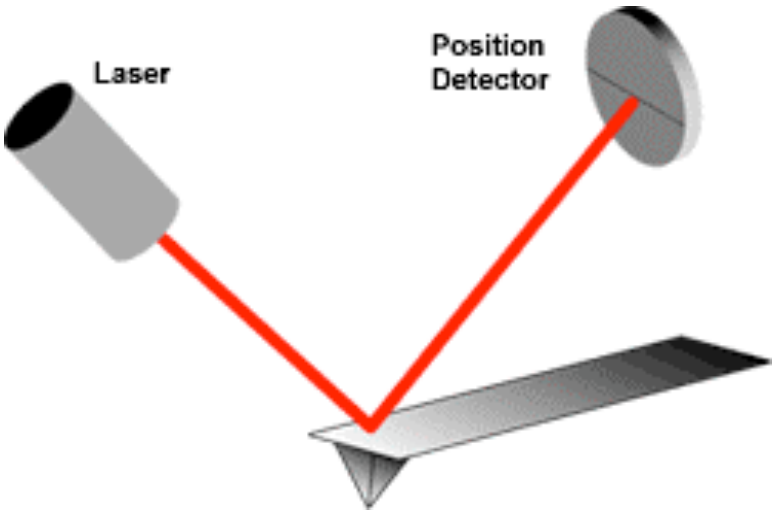


Figure 4.3 Model representing the principles for AFM operation. Laser light from a solid-state diode is reflected off the back of a cantilever-tip assembly and collected by a position sensitive detector (quad photodiode). This detector measures cantilever and feedback electronics to maintain a constant imaging parameter, such as force or tip-sample separation. The sample, which is supported on a solid substrate surface, can be scan in three-dimensions (X, Y, Z) with angstrom precision using a three-axis piezoelectric translation stage. Tip deflections in the order of angstroms, which correspond to forces of approximately 10 pN, can be reliably measured.

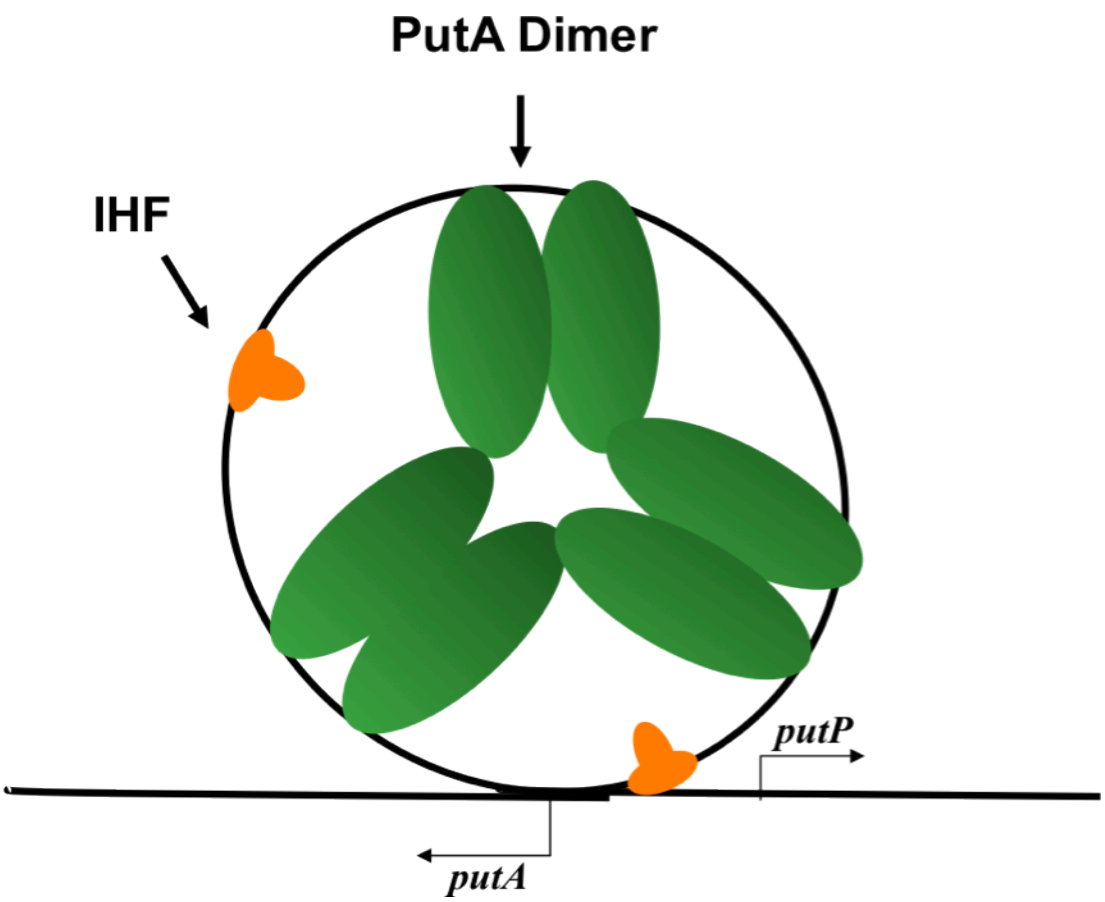


Figure 4.4 Hypothetical Model of the PutAsome. Genetics and biochemical evidence suggest the formation of a higher order structure involved in *put* operon regulation. PutA, IHF and intrinsic bends within this region play an important role in the formation of this structure. We hypothesize protein-protein interactions between PutA proteins and DNA bending by IHF facilitates the formation of a loop that sequesters promoters away from polymerases preventing protein expression.

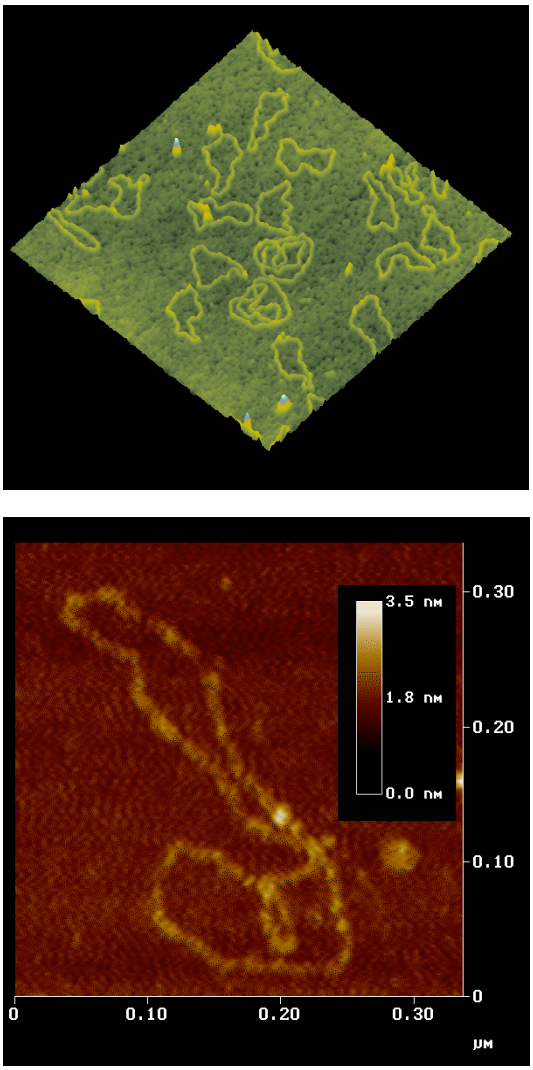


Figure 4.5 Top view of plasmid pBlueconII/*Oput*. AFM was performed on air using SiN tapping mode tip with a spring constant of 0.06 N/m with a resonance frequency of 263.63 kHz. A 0.33 µm surface area was scanned at a 2.4 kHz rate to obtain this single molecule image. DNA was determined to be approximately 2.2 nm in high, consistent with the theoretical helix diameter of 2.0 nm. The irregular pattern observed along the DNA in the lower image represents the helical turn of this particular molecule.

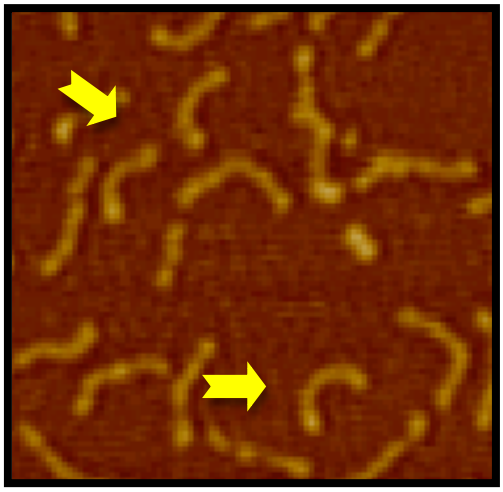
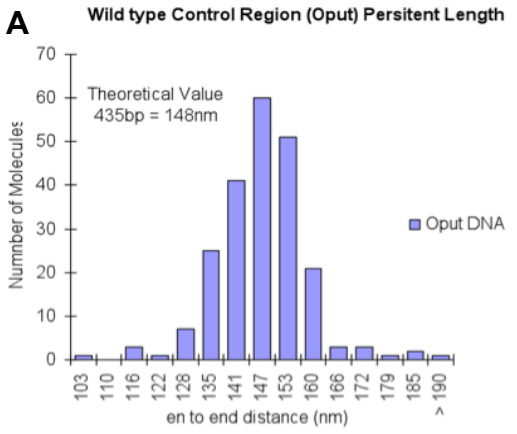
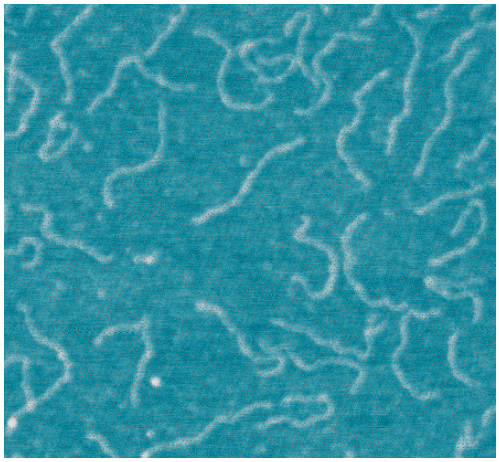


Figure 4.6 *Oput* DNA intrinsic bends. Analysis of DNA molecules bound to mica shows a 435 bp immobilized DNA fragment image in air. Arrows indicate *put* control region DNA fragments exhibiting intrinsic bends.





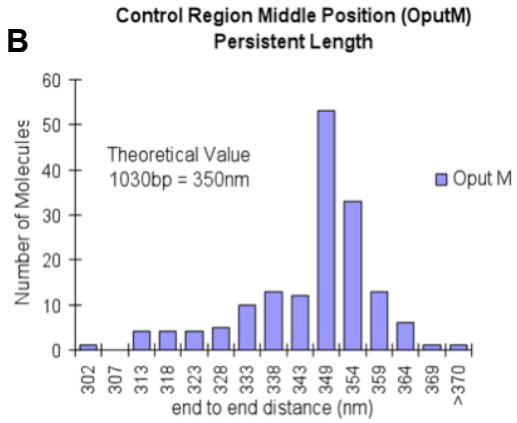


Figure 4.7 *Oput* DNA Persistent Length. DNA persistent lengths are determined by measuring the end-to-end distances of the DNA molecules deposited on mica substrates. Experimentally measure length distributions (panel A & B) can then be used as a reference for measuring DNA in nucleoprotein complexes. Size distribution of the DNA population deposited on mica indicates persistent lengths of approximately 147 nm (~ 432 bp) for a wild type control region fragment. A larger fragment where the control region was located at the middle (*OputM*) resulted in 349 nm (~1,027 bp). Results are close to the theoretical calculated value of approximately 148 nm fragments (435 bp) for *Oput* and 350 nm fragments (1030 bp) for *OputM*.

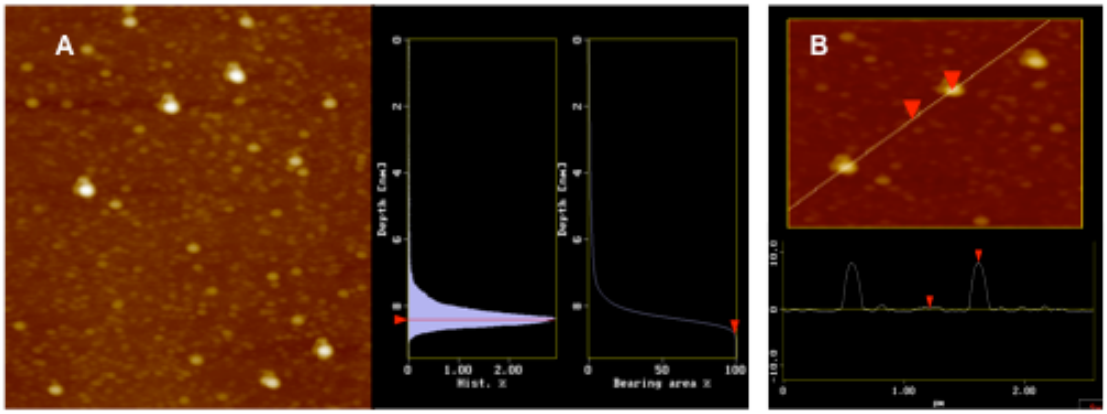


Figure 4.8 Section and bearing analysis for height measurements of deposited PutA protein. An average of these measurements from a universe of molecules in an image was calculated to determine the persistent height in the sample (Panel A). Heights approximate 8 nm, consistent with section analyses where vertical distances show ~8 nm molecules, consistent with previously reported PutA length (Panel B).

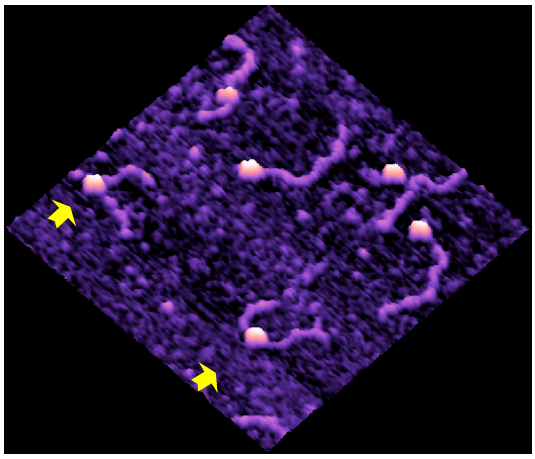


Figure 4.9 DNA bound IHF. DNA bending by IHF proteins bound to one IHF site in the control region DNA was observed (Arrow) by AFM Image analysis.

A B

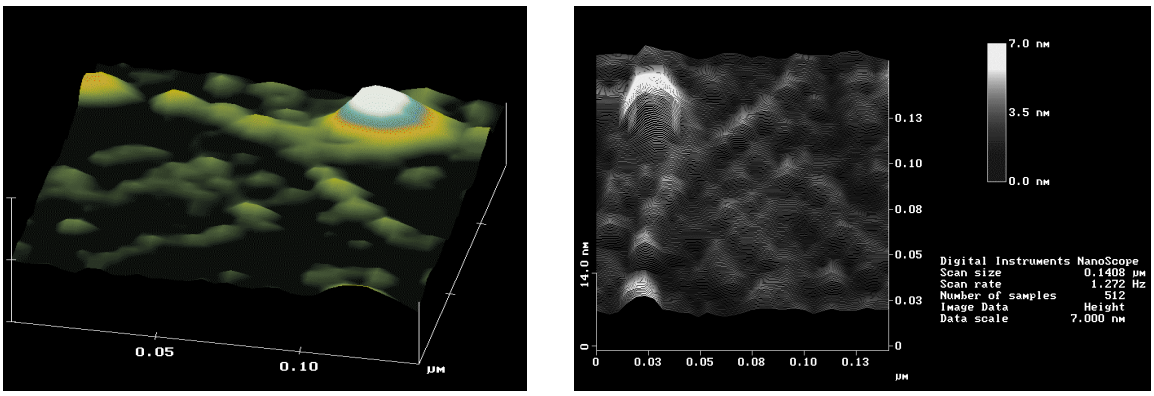


Figure 4.10 Surface plot images of a PutA-IHF-DNA complex. A) A 45˚ surface plot depicting a pyramid like geometry on the protein complex. This image suggests a potential protein-protein interaction by the observed convergence of the proteins at the center of the circumference on the observed complex. B) 90˚ angled plot shows a 7.0 nm height complex with a 223.72 nm DNA fragment wrapped around forming an alpha like structure.

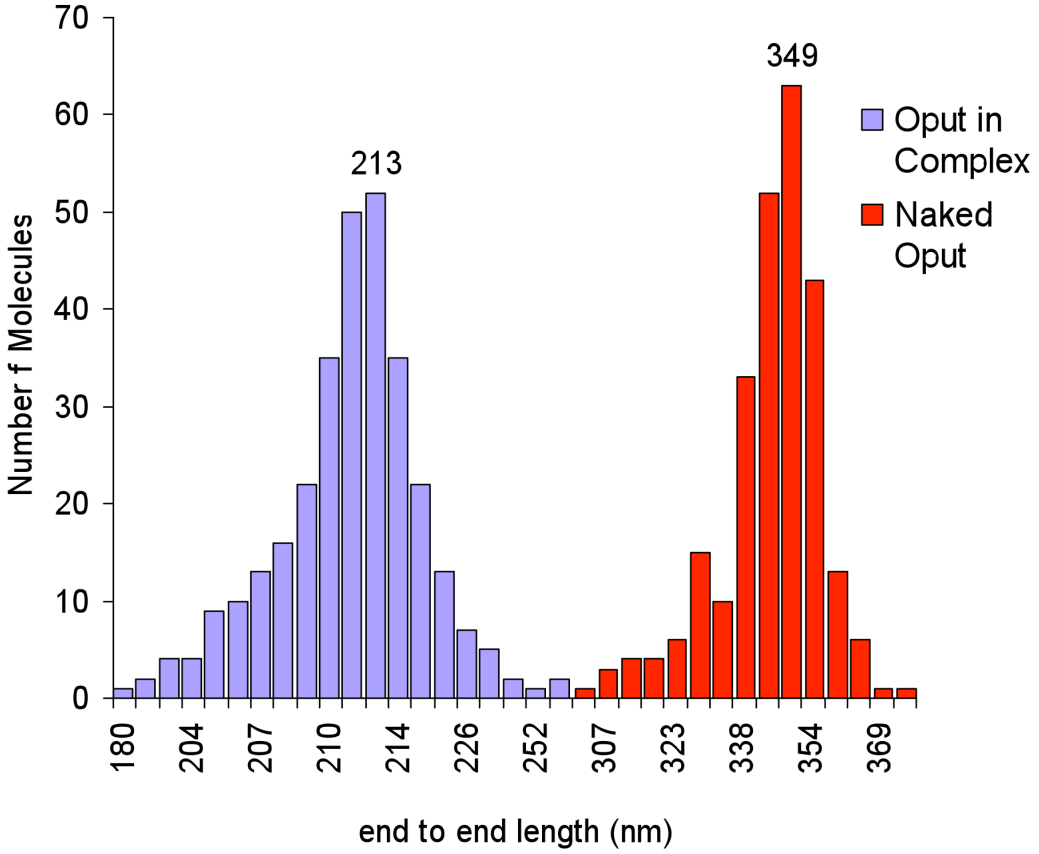


Figure 4.11 PutAsome Complex Sequesters approximately 95% of *put* control region DNA. Naked *Oput* DNA end-to-end distances ranged to 349 nm = 1026 bp. Complex end-to-end distances of unbound DNA ranged to ~ 213 nm = 626 bp ~ 400 bp (95%) inside the PutAsome.

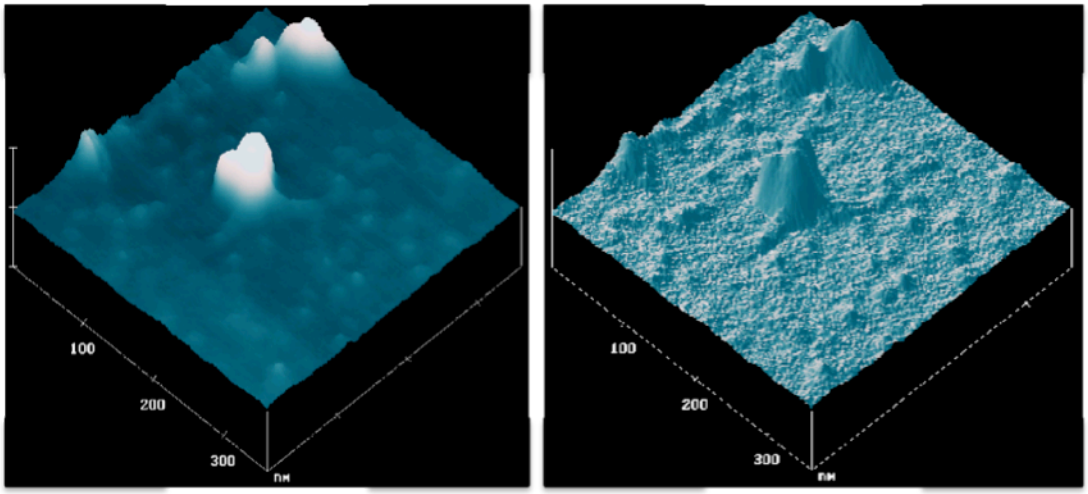


Figure 4.12 Surface plot of PutA and IHF bound Oput DNA. High-resolution surface plot images revealed 3 molecules (right image) potentially interacting at the center of the complex. Consistent with the cooperativity reported in EMSA results from Gomez-Curet, 2001 (13). These images suggest PutA protein interactions that promote the formation and stability of the PutAsome.

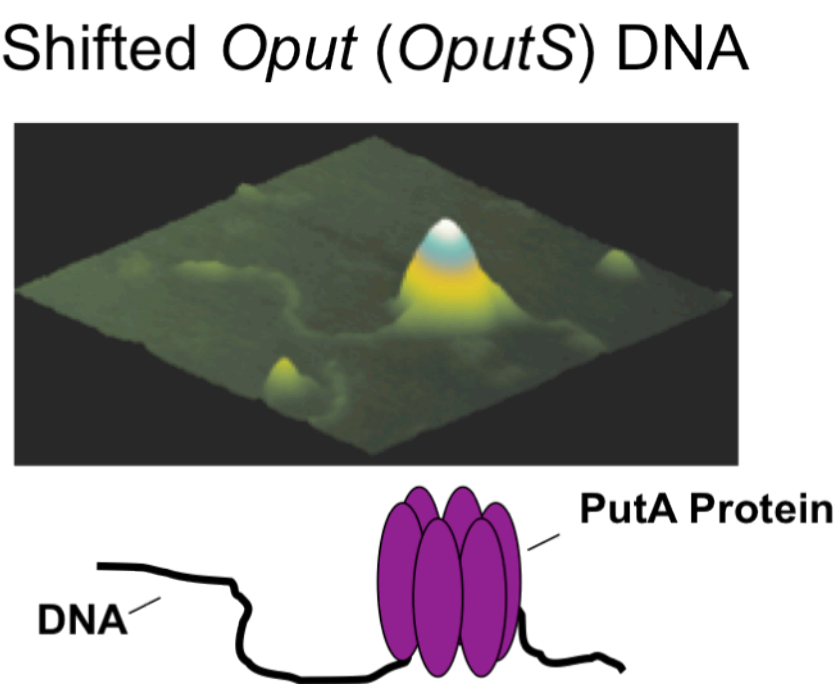


Figure 4.13 Shifted *Oput* region. A 1.03 KB DNA fragments were engineered to shift the put control region to the edges of the DNA molecules. As observed in this image an asymmetrical loop is formed corresponding to the shifted location of the control region in this DNA sequence. The cartoon bellow the image depicts the asymmetric conformation of this structure.

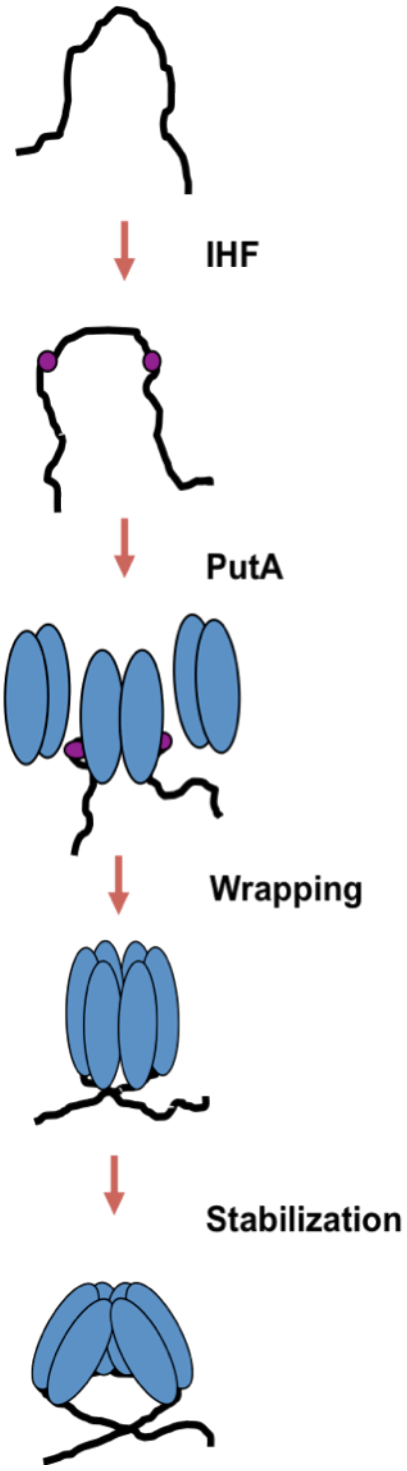


Figure 4.14 Proposed Model the for *put* operon mechanism of repression. PutAsome formation. Structure conformation at the molecular level PutA-IHF-DNA interactions form a higher order structure as originally proposed. However, PutA proteins orientation with respect to the DNA seemed to adopt a perpendicular and not planar conformation. Structure conformation. Alpha-like structure. The PutAsome appears to wrap DNA in an alpha like structure sequestering ~ 95% of control region DNA inside the complex ultimately preventing Polymerase access to *put* operon promoters. PutA multimerization Image analysis suggest protein-protein interactions at the center of the PutAsome. These interactions may stabilize the structure promoting tighter regulation. Moreover, deletion mutants of *put* operator DNA disrupt PutAsome formation.

CURRICULUM VITAE

EDUCATION

Ph.D., Microbiology. University of Illinois at Urbana-Champaign, Urbana, IL (2010)
M.S., Microbiology. University of Illinois at Urbana-Champaign, Urbana, IL (2003)
B.S., Biological Sciences. Universidad del Turabo, Caguas, PR. (1998)

RESEARCH EXPERIENCE

University of Illinois at Urbana-Champaign
Urbana, IL
Research Assistant
2005-present

As active member of the Host Microbe System Theme at the Institute for Genomic biology:

Identified the microbial composition and assessed primates (baboons) as suitable models for women's health.

Did phylogenetic analyses to determine vaginal microbiota structure and relative distribution of species in comparison to humans.

Determined individual-to-individual vaginal microbiota variations in a colony of primates (baboons) using molecular techniques.

Joined efforts to elucidate excision and integration mechanisms and components in important mobilizable elements responsible for the distribution of antibiotic resistance genes across bacterial species.

Initial graduate study focus: (2000-2004)

Studied the autoregulation mechanisms of proline dehydrogenase (PutA) and implemented atomic force microscopy, genetics and biochemical approaches to study protein-DNA complex structures.

Joint Genome Institute/Lawrence Livermore National Laboratory
Walnut Creek, CA
Research Associate
January 2000 - August 2000

Functioned as sequencing team member studying several carbon sequestration microbial species and generated clone libraries for high-throughput sequencing projects.

Used of high-throughput automated equipment aimed at optimizing genome sequences quality and quantity.

Lawrence Livermore National Laboratory
Livermore, CA
Research Assistant
1998–2000

Developed and maintained microcosm apparatus to monitor real time microbial induced corrosion rates and mechanisms.

Collaborated in the development and assembly of a microbial induced corrosion (MIC) cells for polarization resistance data collection to determine microbial induced corrosion rates and material structure impact.

Performed surfacial analysis using scanning electron microscopy and atomic force microscopy to determine microbial induce corrosion on titanium and other metal alloys.

Lawrence Livermore National Laboratory
Livermore, CA
Research Assistant
1996–1997

Designed and directed an environmental microbiology research laboratory.

Tested nuclear repository waste package materials for their susceptibility to MIC and determined rates of biocorrosion under varying environmental conditions.

Characterized microbial isolates from Yucca Mountain nuclear waste repository test site that were suspected to possessed biochemical activities associated with MIC.

Aided in the development of culture media for the isolation of environmental microbial species from Yucca Mountain geologic materials.

TEACHING EXPERIENCE

Department of Microbiology, University of Illinois at Urbana-Champaign, 2007-2010, *Teaching Assistant*. Introductory Microbiology Laboratory Course, MCB 301: Taught introductory experimental microbiology. Experimental microbiology lectures on principles and techniques. Discussed genetics tools and biochemistry of microorganisms.

Department of Microbiology, University of Illinois at Urbana-Champaign, 2005-2007, *Teaching Assistant*. Introductory Microbiology Laboratory Course, MCB 101: Taught basic microbiology sections. Lecture basic laboratory principles and techniques.

PUBLICATIONS

Rivera A. J., Gomez-Curet I., Maloy S. R. Direct Visualization of Proline Utilization Regulatory Complexes by Atomic Force Microscopy. ***In preparation.***

Rivera A. J., Frank J. A., Stumpf R., Salyers A. A., Wilson B., Olsen G., Leigh S. (2010) Unexpected differences between the vaginal microbiota of baboons and humans. *American Journal of Primatology*. ***In press.***

Rivera A. J., Stumpf R., Wilson B., Leigh S., Salyers A. A. (2010) Baboon vaginal microbiota: An overlooked aspect of primate physiology. *American Journal of Primatology.* 72:467-474.

Jeters R., **Rivera A. J.**, Boucek L. M., Stumpf S.M., Leigh S.R., Salyers A.A. (2009) Antibiotic Resistance Genes in the Vaginal Microbiota of Primates Not Normally Exposed to Antibiotics. *Microbial Drug Resistance*. 15(4):309-15.

Horn J. M., Masterson B.A., **Rivera A**., Miranda A., Davis M.A., and Martin S. (2004) Bacterial Growth Dynamics, Limiting Factors and Community Diversity in a Proposed Geological Nuclear Waste Repository Environment. *Geomicrobiology J.* 21(4): 273-286.

Horn J.M., Martin S., **Rivera A**., Bedrossian P. and Tiangan L. (2000) Potential biogenic corrosion of Alloy 22, a candidate nuclear waste packaging material, under simulated repository conditions. Proceedings of Corrosion. *Report UCRL-JC-137269*, *Lawrence Livermore National Laboratory.*

Lian T., Martin S., Jones D., **Rivera A**. and Horn J. (1999) Corrosion of candidate container materials by Yucca Mountain bacteria. *Report UCRL-JC-132825, Lawrence Livermore National Laboratory.*

Horn J., **Rivera A**. and Jones D. (1997) MIC evaluation and testing of the Yucca Mountain repository. *Report UCRL-JC-129198, Lawrence Livermore National Laboratory.*

SYMPOSIA AND PROCEEDINGS

Rivera, A.J., J.F. Frank, R.M. Stumpf, A. A. Salyers, B.A. Wilson, G.J. Olsen, S.R. Leigh. (2010) Unexpected Differences Between the Vaginal Microbiota of Baboons and Humans. Proceedings of the annual meeting of the American Society for Microbiology, abstract N-2711, 110th General Meeting May 23-27, San Diego, CA.

Rivera, A.J., RM Stumpf, M. Ho, S. Sharma, N. Nakamura, S.R. Leigh, B.A. Wilson. (2007) High inter-individual microbial diversity among baboon vaginal ecosystems. AAPA Conference, Philadelphia, PA.

Rivera A.J., J. Frank, R.M. Stumpf, S.R. Leigh, G. Olsen, B.A. Wilson. 2007 Microbial patterns of diversity among baboon vaginal ecosystems. 4th Annual Midwest Primate Interest Group Conference, Carbondale, IL.

Rivera A.J., Frank J. A. Stumpf R. Leigh S., Wilson B., Reich C., Olsen G., Salyers A. 2007. A preliminary look at high inter-individual microbial diversity among baboon vaginal ecosystems. Proceeding of the annual meeting of the American Society for Microbiology abstract N-112, 107th General Meeting May 21-25, Toronto CA.

Rivera A.J. and S. Maloy. 2006. Imaging the PutAsome: Structural determination using Atomic Force Microscopy. Proceedings of the annual meeting of the American Society for Microbiology, Abstract H-110, 106th General Meeting, May 21-25, Orlando, FL.

Rivera A. J. and S. Maloy. 2005. Structural determination of the PutAsome Using Atomic Force Microscopy and Two-Hybrid System. Proceeding of the annual meeting of the American Society for Microbiology, Abstract H-042, 105th General Meeting, June 5-9, Atlanta, GA.

Rivera A. J. and S. Maloy. 2005. The PutAsome: Seeing is Believing. Proceedings of the Molecular Genetics of Bacteria and Phage Meeting, August 2-7, Madison, Wisconsin.

Rivera, A.J. and S. Maloy. 2003. The PutAsome: Seeing is Believing. Proceedings of the Annual meeting of the American Society for Microbiology, Abstract H-94, 103rd General Meeting, May 18-22, Washington DC, MD.

Horn, J.M., M. Davis, B. Masterson, **A. Rivera**, A. Miranda, and S. Martin. 1999. Bacterial growth and nutrient availability in a subsurface nuclear waste repository environment, Proceedings of the Annual Meeting of the American Society of Microbiology, Abstract Q-168, 99th General Meeting, May 30-June3, Chicago, IL.

Martin, S., B. Masterson, **A. Rivera**, M. Miranda, T. Lian, and J.M. Horn. 1999. Biochemical contributions to corrosion of nuclear waste container materials, Proceedings of the Annual Meeting of the American Society of Microbiology, Abstract Q-374, 99th General Meeting, May 30-June 3, Chicago, IL.

MEMBERSHIPS, AWARDS AND APPOINTMENTS

Recipient of the University of Illinois Department of Microbiology 2009 Francis and Harlie Clark Graduate Teaching Assistantship Award.

Consistently ranked, “Outstanding and Excellent Teaching Assistant” for Introductory Experimental Microbiology (MCB 101) and Experimental Microbiology (MCB 301) from fall 2005 to present.

American Society for Microbiology CRP Travel Fellowship 105th General Meeting May 21- 25, 2005. Atlanta, GA.

Member of the American Society for Microbiology since 1999.

Received a Magna Cum Laude distinction from Universidad del Turabo, 1998.

Member of the Science and Engineering Honor Program, Systema Ana G. Mendez, Universidad del Turabo.